In re patent application of Aviv Shaish

Serial No. 10/668,601 Group Art Unit: 1654

Filed: 09/24/2003 Examiner: M.C. Flood

For: THERAPEUTIC USES OF DUNALIELLA POWDER

DECLARATION
under Rule 132

Commissioner of Patents and Trademarks
Washington, D.C. 20231

I, Ami Ben-Amotz, an Israeli citizen residing at 21 Hatomer St., Savyon, Israel, hereby declare:

1. I am currently Senior Investigator, The National Institute of Oceanography, Israel Oceanographic & Limnological Research, Haifa, Israel.

2. My list of publications is attached herewith as **Annex A**. My fields of expertise include: Algal biochemistry; Carotenoids biosynthesis; Separation and identification of carotenoids and retinoids.

3. I am familiar with the above captioned application (hereinafter: *"the application"*), and with the claims thereof.

4. I am one of the authors in each of the following articles:

   (a) Levy, Y. et al, *Dietary supplementation of a natural isomer mixture of beta-carotene inhibits oxidation of LDL derived from patients with diabetes mellitus* (2000) Ann Nutr Metab 44:54-60 [hereinafter: "*Levy (U)*"];

   (b) Levy, Y. et al, *Effect of dietary supplementation of different β-carotene isomers on lipoprotein oxidative modification* (1995) J Nutr Envir Med 5:13-22 [hereinafter; "*Levy (W)*"].

5. Both of the Levy articles are based on the hypothesis that atherogenesis involves oxidative modification of low density lipoprotein (LDL), which is associated with the depletion of the LDL endogenous anti-oxidants, and that enrichment of LDL with the anti-oxidant β-carotene has the potential of reducing the susceptibility of LDL to lipid peroxidation. In other words, LDL is protected

against oxidation by anti-oxidants, and the β-carotene contained in Dunaliella acts as an anti-oxidant.

6. Levy (W) was published in 1995 and concludes that β-carotene prevents artherogenesis by acting as an anti-oxidant (abstract, last 2 sentences; discussion, 1st paragraph). Levy (U) was published in 2000 and concludes that β-carotene normalizes enhanced LDL-oxidation and therefore suggests a therapeutic role to different antioxidants in diabetes (abstract, conclusions; page 59, last paragraph).

7. However, in recent years, and certainly by the filing date of the application (late 2003), this hypothesis had been proven to be in error. I enclose a number of articles describing studies carried out which disprove the above hypothesis.

8. Yusuf, S. et al, Vitamin E supplementation and cardiovascular events in high-risk patients, New England Journal of Medicine (2000) 342:154-60 [**Annex B**], and Hegele, R.A., ACE inhibition in the secondary prevention of vascular disease: the Heart Outcomes Prevention Evaluation (HOPE) trial and its substudies, Current Atherosclerosis Reports (2000) 2:361-362 [**Annex C**], both describe the results of a large scale (over 9000 patients) clinical study of patients at high risk for cardiovascular events because they had cardiovascular disease or diabetes in addition to one other risk factor. Selected patients were treated for 4.5 years with vitamin E, a known anti-oxidant. The study concluded that vitamin E had no apparent effect on cardiovascular outcomes (Annex B, abstract, conclusions; Annex C, discussion).

9. Kritharides, L. and Stocker, R., The use of antioxidant supplements in coronary heart disease, Atherosclerosis (2002) 164:211-219 [**Annex D**], is an article which reviewsrandomised controlled studies investigating the clinical use of antioxidant supplements to prevent or treat coronary heart disease (CHD). The article concludes that although in the past, various studies have been interpreted as supporting a role for antioxidants in the prevention of CHD, "supplements of α-tocopherol [= vitamin E – *aba*] and β-carotene cannot be recommended for the treatment or prevention of CHD" (abstract).

10. Zureik, M. et al, Effects of long-term daily low-dose supplementation with antioxidant vitamins and minerals on structure and function of large arteries, Arterioscler. Thromb. Vasc. Biol. (2004) 24:1485-1491 [**Annex E**], describes the results of a study carried out on 1162 subjects in France during a period of 7.5

years. The anti-oxidants taken by the subjects included β-carotene. The conclusion reached was that of "no beneficial effects of long-term daily low-dose supplementation of antioxidant vitamins and minerals on carotid atherosclerosis and arterial stiffness" (abstract, conclusion).

11. In summary, at the date of the filing of the application, it had become clear that the hypothesis on which the Levy articles were based had been proven incorrect, and the articles had been shown to be in error with respect to the use of β-carotene obtained from crude Dunaliella powder as an antioxidant in the treatment of diabetes mellitus and atherosclerosis. In view of the findings that antioxidants lack efficacy in the treatment of cardiovascular disease, scientists working in the field would disregard the conclusions of the Levy articles.

12. The invention described in the application is thus surprising and unexpected in showing that the β-carotene contained in Dunaliella powder apparently differs from the β-carotene used in the aforementioned studies, and is effective in the treatment of diabetes mellitus and atherosclerosis.

13. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Sept 5, 2004 Ami Ben-Amotz

Prof. Ami Ben-Amotz

ANNEX A

CURRICULUM VITAE
AMI BEN-AMOTZ

Date of birth and place: 30 July 1943, Tel-Aviv, Israel

EDUCATION AND EXPERIENCE

Jan 1998-	Elected member of the board, International Society of Applied Phycology, Member, International Society of Carotenoids, Member, American Phycological Society, Elected member, New York Academy of Sciences, USA
Jan 1997-	Advisor, United States Department of Agriculture
Jan. 1990-	Chief Scientist, *Dunaliella*, N.B.T. Ltd., Eilat, Israel.
Jan. 1992	Coordinator Professor, *Dunaliella* Club, the Weizmann Institute of Science, Rehovot, Israel.
1986- 2002	Advisor, Nikken Sohonsha Co., Gifu, Japan
Apr. 1986-	Research Professor, National Institute of Oceanography, Israel Oceanographic & Limnological Research, Haifa, Israel.
Jan. 1984-	Head, Dept. of Marine Biology, National Institute of Oceanography,
Jan. 1991	Israel Oceanographic & Limnological Research, Haifa, Israel.
Oct. 1980-	Associate Research Professor, National Institute of Oceanography,
Apr. 1986	Israel Oceanographic &Limnological Research, Haifa, Israel.
Jul. 1976-	Consultant, *Dunaliella* project, The Weizmann Institute of
Jan. 1990	Science, Rehovot, Israel; Adviser, *Dunaliella* project, Koor Foods Ltd.
Jul. 1982-	Visiting Professor, School of Applied Biology, Georgia Institute
Aug. 1983	of Technology, Atlanta, GA, U.S.A.
Jan. 1976-	Senior Biologist, National Institute of Oceanography, Israel
Oct. 1980	Oceanographic & Limnological Research, Haifa, Israel.
Sep. 1975-	Assistant Professor, Botany Dept., The Hebrew University of
Sep. 1976	Jerusalem, Israel.
Sep. 1975-	Lady Davis Fellow, awarded by the Lady Davis Fellowship Trust,
Jan. 1976	Jerusalem, Israel. Visiting Scientist, Scripps Institution of Oceanography, La Jolla, California, U.S.A.
Sep. 1973-	Postdoctoral fellow, Dept. of Biology, Brandeis University,
Aug. 1975	Waltham, Massachusetts, U.S.A., with Prof. M. Gibbs.
Jul. 1973	Investigator in a summer research program of Experimental
Jul. 1974	Marine Botany, Marine Biological Laboratory, Woods Hole, Massachusetts, U.S.A.
Jun. 1973	The Weizmann Institute of Science, Rehovot, Israel, Ph.D. Biochemistry. Thesis title: "Photosynthetic and osmoregulation mechanism in the halophilic alga *Dunaliella parva.*" under the supervision of Prof. M. Avron.
Jun. 1969	The Hebrew University of Jerusalem, Israel, M.Sc. Biochemistry ~ with distinction, under the supervision of Profs. I. Ohad and B.Z. Ginzburg.

PUBLICATIONS

Ami BEN-AMOTZ

1. Ben-Amotz, A. and Ginzburg, B.Z. (1969)
Light-induced proton uptake in whole cells of *Dunaliella parva.*
Biochim. Biophys. Acta 183: 144-154.

2. Ben-Amotz, A. and Avron, M. (1972)
Photosynthetic activities of the halophilic alga *Dunaliella parva.*
Plant Physiol. 49: 240-243.

3. Ben-Amotz, A. and Avron, M. (1972)
Is NADP an obligatory intermediate in photosynthesis.
Plant Physiol. 49: 244-248.

4. Ben-Amotz, A. and Avron, M. (1973)
The role of glycerol in the osmotic regulation of the halophilic alga *Dunaliella parva.*
Plant Physiol. 51: 875-878.

5. Ben-Amotz, A. and Avron, M. (1973)
NADP specific dihydroxyacetone reductase from *Dunaliella parva.*
FEBS Lett. 29: 153-155.

6. Ben-Amotz, A. (1974)
Osmoregulation mechanism in the halophilic alga *Dunaliella parva.*
In: Membrane Transport in Plants (Eds.U. Zimmerman and J. Dainty), Springer-Verlag, Berlin-Heidelberg-New York, pp. 95-100.

7. Ben-Amotz, A. and Avron, M. (1974)
isolation, characterization and partial purification of an $NADP^+$-dependent dihydroxyacetone reductase from the halophilic alga *Dunaliella parva.*
Plant Physiol. 53: 628-631.

8. Gibbs, M., Bamberger, E.S., Ben-Amotz, A., Ehrlich, B.A. and Erbes, D.L. (1974).
The chloroplast and reducing equivalents.
Portugaliae Acta Biologica 14: 30-44.

9. Ben-Amotz, A. (1975)
Adaptation of the unicellular alga *Dunaliella parva* to a saline environment.
J. Phycol. 11: 50-54.

10. Ben-Amotz, A., Erbes, D.L., Henderson, M.A., Peavey, D.G. and Gibbs, M. (1975)
H_2 metabolism in photosynthetic organisms. I. Dark H_2 evolution and uptake by algae and mosses.
Plant Physiol. 56: 72-77.

11. Ben-Amotz, A. and Gibbs, M. (1975)
H_2 metabolism in photosynthetic organisms. II. Light-dependent H_2 evolution by preparations of *Chlamydomonas, Scenedesmus* and spinach.
Biochem. Biophys. Res. Communs. 64: 355-359.

12. King, D., Erbes, D.L., Ben-Amotz, A. and Gibbs, M. (1976)
H_2 metabolism in photosynthetic organisms, mechanism of H_2 photoevolution.
In: Research in Photobiology (Ed. A. Castellani), Plenum Press, New York, pp. 329-335.

13. King, D., Erbes, D.L., Ben-Amotz, A. and Gibbs, M. (1977)
The mechanism of hydrogen photoevolution in photosynthetic organisms.

In: Biological Solar Energy Conversion (Eds. A. Mitsui, S. Miyachi, A. San Pietro and S. Tamura), Academic Press, New York, San Francisco, London, pp. 69-75.

14. Ben-Amotz, A. and Avron, M. (1978)
On the mechanism of osmoregulation in *Dunaliella*.
In: Energetics and Structure of Halophilic Microorganisms (Eds. S.R. Caplan and M. Ginzburg), Elsevier, pp. 529-541.

15. Avron, M. and Ben-Amotz, A. (1979)
Metabolic adaptation of the alga *Dunaliella* to low water activity.
In: Strategies of Microbial Life in Extreme Environments (Ed. M. Shilo), Dalem Konferenzen, Verlag Chimie, Weinheim, New York, pp. 83-91.

16. Ben-Amotz, A. (1979)
Hydrogen metabolism.
In: Encyclopedia of Plant Physiology, New Series (Eds. M. Gibbs and E. Latzko), Springer-Verlag, Berlin-Heidelberg-New York, pp. 497-505.

17. Gilboa, A. and Ben-Amotz, A. (1979)
An improved method for rapid assaying of viability of cryopreserved unicellular algae.
Plant Sci. Lett. 14: 317-320.

18. Ben-Amotz, A. and Avron, M. (1980)
Glycerol, β-carotene and dry algal meal production by *Dunaliella*.
In: Algal Biomass: Production and Use (Eds. G. Shelef and C.J. Soeder), Elsevier, Amsterdam, pp. 603-610.

19. Ben-Amotz, A. and Avron, M. (1980)
Osmoregulation in the halophilic algae *Dunaliella* and *Asteromonas*.
In: Basic Life Sciences Series, Vol. 14. Genetic Engineering of Osmoregulation: Impact on Plant Productivity for Food, Chemicals and Energy (Eds. D.W. Rains, R.C. Valentine and A. Hollaender), Plenum Press, New York, London, pp. 91-99.

20. Ben-Amotz, A. (1980)
Glycerol production in the alga *Dunaliella*.
In: Biochemical and Photosynthetic Aspects of Energy Production (Ed. A. San Pietro), Academic Press, New York, pp. 191-208.

21. Hase, T., Matsubara, H., Ben-Amotz, A., Rao, K.K. and Hall, D.O. (1980)
Purification and sequence of two ferredoxins from *Dunaliella salina*.
Phytochem. 19: 2065-2070.

22. Ben-Amotz, A. and Gilboa, A. (1980)
Cryopreservation of marine unicellular algae. I. A survey of algae with regard to size, culture age, photosynthetic activity and chlorophyll-to-cell ratio.
Mar. Ecol. 2: 157-161.

23. Ben-Amotz, A. and Gilboa, A. (1980)
Cryopreservation of marine unicellular algae. II. Induction of freezing tolerance.
Mar. Ecol. 2: 221-224.

24. Ben-Amotz, A. and Avron, M. (1980)
Algae, plant potential which has not yet been exploited. (in Hebrew)
Mada 24: 18-20.

25. Wegmann, K., Ben-Amotz, A. and Avron, M. (1980)
The effect of temperature on glycerol retention in the halotolerant algae *Dunaliella* and *Asteromonas*.
Plant Physiol. 66: 1196-1197.

26. Ben-Amotz, A. and Grunwald, T. (1981)
Osmoregulation in the halotolerant alga *Asteromonas gracilis.*
Plant Physiol. 67: 613-616.

27. Ben-Amotz, A. and Avron, M. (1981)
Glycerol and β-carotene metabolism in the halotolerant alga *Dunaliella*: a model system for biosolar energy conversion.
Trends in Biochem. Sci. 6: 297-299.

28. Ben-Amotz, A. and Rosenthal, H. (1981)
Cryopreservation of marine unicellular algae and early life stages of fish for use in mariculture.
Europ. Maricult. Soc. Spec. Publ. 6: 149-162.

29. Ben-Amotz, A. and Avron, M. (1982)
The potential use of *Dunaliella* for the production of glycerol and carotene.
In: Biosaline Research: A Look to the Future (Ed. A. San Pietro), Plenum Press, New York, pp. 207-214.

30. Ben-Amotz, A., Sussman, I. and Avron, M. (1982)
Glycerol production by *Dunaliella.*
Experientia 38: 49-52.

31. Ben-Amotz, A. and Fishler, R. (1982)
Induction of sexual reproduction and resting egg production in *Brachionus plicatilis* by a diet of salt-grown *Nannochloris oculata.*
Mar. Biol. 67: 289-294.

32. Ben-Amotz, A., Katz, A. and Avron, M. (1982)
Accumulation of β-carotene in halotolerant algae: purification and characterization of β-carotene globules from *Dunaliella bardawil* (Chlorophyceae).
J. Phycol. 18: 529-537.

33. Fried, A., Tietz, A., Ben-Amotz, A. and Eichenberger, W. (1982)
Lipid composition of the halotolerant alga *Dunaliella bardawil.*
Biochim. Biophys. Acta 713: 419-426.

34. Ben-Amotz, A. and Avron, M. (1983)
On the factors which determine massive β-carotene accumulation in the halotolerant alga *Dunaliella bardawil.*
Plant Physiol. 72: 593-597.

35. Ben-Amotz, A. and Avron, M. (1983)
Accumulation of metabolites by halotolerant algae and its industrial potential.
Ann. Rev. Microbiol. 37: 95-119.

36. Rebhun, S. and Ben-Amotz, A. (1984)
The distribution of cadmium between the marine alga *Chlorella stigmatophora* and seawater medium. Effect on algal growth.
Water Res. 18: 173-175.

37. Ben-Amotz, A. (1984)
Production of nutritional microphytoplankton for use as food in marine fish hatcheries.
In: Research on Aquaculture (Eds. H. Rosenthal and S. Sarig). European Maricult. Soc. Spec. Publ. 8: 195-200.

38. Ben-Amotz, A., Thomas, W.H. and Tornabene, T.G. (1985)
Chemical profile of selected species of unicellular algae with emphasis on lipids and fatty acids.
J. Phycol. 21: 72-81.

39. Ben-Amotz, A. (1985)
Algae in salt water.

In: Plants and Animals in Israel. Society for the Protection of Nature in Israel, pp. 201-204.

40. Tornabene, T.G., Bourne, T.F., Raziudin, S. and Ben-Amotz, A. (1985)
Lipid and lipopolysaccharide constituents of cyanobacterium *Spirulina platensis*.
Mar. Ecol. Prog. Ser. 22: 121-125.

41. Ben-Amotz, A., Edelstein, S. and Avron, M. (1986)
Use of β-carotene rich alga *Dunaliella bardawil* as a source of vitamin A.
British Poultry Sci. 27: 613-619.

42. Rebhun, S. and Ben-Amotz, A. (1986)
Effect of NaCl concentration on cadmium uptake by the halophilic alga *Dunaliella salina*.
Mar. Ecol. Prog. Ser. 30: 215-219.

43. Pick, U., Ben-Amotz, A., Karni, L., Seebergts, C.J. and Avron, M. (1986)
Partial characterization of K^+ and Ca^{++} uptake systems in the halotolerant alga *Dunaliella salina*.
Plant Physiol. 81: 875-881.

44. Ben-Amotz, A. (1986)
β-carotene enhancement and its role in protecting *Dunaliella bardawil* against injury by high irradiance.
Beih. Nova Hedwigia 83: 132-135.

45. Ben-Amotz, A., Gressel, J. and Avron, M. (1987)
Massive accumulation of phytoene induced by norflurazon in *Dunaliella bardawil* (Chlorophyceae) prevents recovery from photoinhibition.
J. Phycol. 23: 176-181.

46. Ben-Amotz, A., Fishler, R. and Schneller, A. (1987)
The chemical composition of dietary species of marine unicellular algae and rotifers with emphasis on fatty acids.
Mar. Biol. 95: 31-36.

47. Ben-Amotz, A. (1987)
Effect of irradiance and nutrient deficiency on the chemical composition of *Dunaliella bardawil* Ben-Amotz & Avron (Volvocales, Chlorophyceae).
J. Plant Physiol. 131: 479-487.

48. Rebhun, S. and Ben-Amotz, A. (1988)
The antagonistic effect of manganese to cadmium toxicity in the alga *Dunaliella salina*.
Mar. Ecol. Prog. Ser. 42: 97-104.

49. Ben-Amotz, A., Mokady, S. and Avron, M. (1988)
The β-carotene rich alga *Dunaliella bardawil* as a source of retinol in rat diet.
Brit. J. Nutr. 59: 443-449.

50. Gaba, V., Cohen, N., Shaltiel, Y., Ben-Amotz, A. and Gressel, J. (1988)
Light-requiring acifluorfen action in the absence of bulk photosynthetic pigments.
Pesticides Biochem. Physiol. 31: 1-12.

51. Ben-Amotz, A., Lers, A. and Avron, M. (1988)
Stereoisomers of β-carotene and phytoene in the alga *Dunaliella bardawil*.
Plant Physiol. 86: 1286-1291.

52. Yamaguchi, K., Kawamata, M., Murakami, M., Konosu, S. and Ben-Amotz, A. (1988)
Extractive nitrogenous components of the haotolerant alga *Dunaliella bardawil*.
Nippon Suisan Gakkaishi 54: 239-243.

53. Boussiba, S., Sandbank, E., Shelef, G., Cohen, Z., Vonshak, A., Ben-Amotz, A., Arad, S. and Richmond, A. (1988)
Outdoor cultivation of the marine microalga *Isochrysis galbana* in open reactors.
Aquaculture 72: 247-253.

54. Ben-Amotz, A. and Avron, M. (1989)
The wavelength dependence of massive carotene synthesis in *Dunaliella bardawil* (Chlorophyceae).
J. Phycol., 25: 175-178.

55. Nagasawa, H., Konishi, R., Sensui, N., Yamamoto, K. and Ben-Amotz, A. (1989)
Inhibition by β-carotene rich algae *Dunaliella* of spontaneous mammary tumorigenesis in mice.
Anticancer Res. 9: 71-76.

56. Nagasawa, H., Konishi, R., Yamamoto, K. and Ben-Amotz, A. (1989)
Effects of natural β-carotene on production and body growth in mice.
In Vivo, 3: 79-82.

57. Ben-Amotz, A., Mokady, S., Edelstein, S. and Avron, M. (1989)
Bioavailability of a natural isomer mixture as compared with synthetic all-trans β-carotene in rats and chicks.
J. Nutr., 119: 1013-1019.

58. Ben-Amotz, A. and Avron, M. (1989)
The biotechnology of mass culturing *Dunaliella* for products of commercial interest.
In: Algal and Cyanobacterial Biotechnology. (Eds. R.C. Cresswell, T.A.V. Rees and N. Shah). Longman Scientific & Technical, New York, pp. 91-114.

59. Nagasawa, H., Fujii, Y., Yamamoto, K., Konishi, R. and Ben-Amotz, A. (1989)
No deleterious side effects on mammary growth and endocrine parameters of chronic ingestion of β-carotene rich alga *Dunaliella bardawil* in virgin mice in comparison with synthetic all-trans β-carotene.
The Cancer J., 2: 391-394.

60. Ben-Amotz, A., Shaish, A. and Avron, M. (1989)
The mode of action of the massively accumulated β-carotene of *Dunaliella bardawil* in protecting the alga against damage by excess irradiation.
Plant Physiol., 91: 1040-1043.

61. Ben-Amotz, A. and Avron, M. (1990)
The beta-carotene accumulating halotolerant alga *Dunaliella*: A new venture in biotechnology.
Biotech. Forum Europe, 7: 48-53.

62. Ben-Amotz, A. and Avron, M. (1990)
The biotechnology of cultivating the halotolerant alga *Dunaliella.*
Trends in Biotechnology, 8: 121-126.

63. Mokady, S., Avron, M. and Ben-Amotz, A. (1990)
Accumulation in chick livers of 9-cis versus all-trans β-carotene.
J. Nutr., 120: 889-892.

64. Shaish, A., Avron, M. and Ben-Amotz, A. (1990)
Effect of inhibitors on the formation of stereoisomers in the biosynthesis of β-carotene in *Dunaliella bardawil.*
Plant Cell Physiol., 31: 689-696.

65. Friedlander, M. and Ben-Amotz, A. (1990)
Acclimation of brown seaweeds in an outdoor cultivation system and their cytokinin-like activity.
J. Appl. Phycol., 2: 145-154.

66. Shlomai, P., Ben-Amotz, A. and Margalith, P. (1991)

Production of carotene stereoisomers by *Phycomyces blakesleeanus*.
Appl. Microbiol. Biotechnol., 34: 458-462.

67. Shlomai, P., Ben-Amotz, A. and Margalith, P. (1991)
The effect of veratrole on carotenoid biosynthesis by *Phycomyces blakesleeanus*.
J. Appl. Bacteriol., 70: 166-168.

68. Mokady, S. and Ben-Amotz, A. (1991)
Dietary lipid level and the availability of β-carotene of *Dunaliella bardawil* in rats.
Nutr. Cancer, 15: 47-52.

69. Friedlander, M. and Ben-Amotz, A. (1991)
The effect of outdoor culture conditions on growth and epiphytes of *Gracilaria conferta*.
Aquat. Bot., 39: 315-333.

70. Shaish, A., Ben-Amotz, A. and Avron, M. (1991)
Production and selection of high β-carotene mutants of *Dunaliella bardawil* (Chlorophyta).
J. Phycol., 27: 652-656.

71. Friedlander, M., Krom, M.D. and Ben-Amotz, A. (1991)
The effect of light and ammonium on growth, epiphytes and chemical constituents of *Gracilaria conferta* in outdoor cultures.
Bot. Mar., 34: 161-166.

72. Lavy, A., Brook G.J., Dankner, G., Ben-Amotz, A. and Aviram, M. (1991)
Enhanced *in vitro* oxidation of plasma lipoproteins derived from hypercholesterolemic patients.
Metabolism, 40: 794-799.

73. Ben-Amotz, A., Shaish, A. and Avron, M. (1991)
The biotechnology of cultivating *Dunaliella* for production of β-carotene rich algae.
Bioresource Technol. 38: 233-235.

74. Nagasawa, H., Fujii, Y., Kageyama, Y., Segawa, T. and Ben-Amotz, A. (1991)
Suppression by beta-carotene rich algae *Dunaliella bardawil* of the progression, but not the development, of spontaneous mammary tumours in SHN virgin mice.
Anticancer Res. 11: 713-718.

75. Avron, M. and Ben-Amotz, A. (eds.) (1992)
Dunaliella: Physiology, Biochemistry and Biotechnology.
CRC Press, Florida, 240 pp.

76. Shaish, A., Ben-Amotz, A. and Avron, M. (1992)
Biosynthesis of β-carotene in *Dunaliella*.
Methods in Enzymol. 213: 439-444.

77. Arad (Malis), S., Cohen, E. and Ben-Amotz, A. (1993)
Accumulation of cantaxanthin in *Chlorella emersonii*.
Physiol. Plant. 87: 232-236.

78. Shaish, A., Avron, M., Pick, U. and Ben-Amotz. A. (1993)
Are active oxygen species involved in induction of β-carotene in *Dunaliella bardawil*?
Planta 190: 363-368.

79. Lavy, A., Ben-Amotz, A. and Aviram, M. (1993)
Preferential inhibition of LDL oxidation by all-trans and 9-cis β-carotenes.
Eur. J. Clin. Chem. Clin. Biochem. 31: 83-90.

80. Fujii, Y., Sakamoto, S., Ben-Amotz, A. and Nagasawa, H. (1993)
Effects of beta-carotene-rich algae *Dunaliella bardawil* on the dynamic changes of normal and neoplastic mammary cells and general metabolism in mice.
Anticancer Res. 13: 389-393.

81. Ben-Amotz, A. (1993)
The pioneering biotechnological path of the late Prof. Avron, *Dunaliella*: (Volvocales, Chlorophyta); from glycerol to β-carotene.
Jpn. J. Phycol. (Sorui) 41: 373-378.

82. Takenaka, H., Takahashi, H., Hayashi, K. and Ben-Amotz, A. (1993)
Protective effect of *Dunaliella bardawil* on water-immersion-induced stress in rats.
Planta Med. 59: 421-424.

83. Levin, G., Ben-Amotz, A. and Mokady, S. (1993)
Dietary synthetic β-carotene or *Dunaliella bardawil* and hepatic lipid peroxidation.
J. Opt. Nutr. 2: 143-150.

84. Levy, Y., Ben-Amotz, A., Dankner, G., Brook, J.G. and Aviram, M. (1993)
Enhanced lipid peroxidation of low density lipoprotein from subjects supplemented with fish oil.
J. Opt. Nutr. 2: 6-9.

85. Levin, G., Ben-Amotz, A. and Mokady, S. (1994)
Liver accumulation of soluble all-trans or 9-cis β-carotene in rats and chicks.
Comp. Biochem. Physiol. 107A: 203-207.

86. Bitterman, N., Melamed, Y. and Ben-Amotz, A. (1994)
β-carotene and CNS oxygen toxicity in rats.
J. Appl. Physiol. 76: 1073-1076.

87. Levy, Y., William, N., Omensky, T., Ben-Amotz, A. and Cohen, A. (1994)
Relationship between plasma antioxidants and coronary artery disease (in Hebrew).
Harefua (The Medicine) 127: 154-157.

88. Lavy, A., Fuhrman, B., Markel, A., Dankner, G., Ben-Amotz, A., Presser, D. and Aviram, M. (1994)
Effect of dietary supplementation of red or white wine on human blood chemistry, nematology and coagulation; favorable effect of red wine on plasma high-density lipoprotein.
Ann. Nutr. Metab. 38: 287-294.

89. Ben-Amotz, A. (1995)
New mode of *Dunaliella* biotechnology: two phase growth for β-carotene production.
J. Appl. Phycol. 7: 65-68.

90. Levy, Y., Ben-Amotz, A. and Aviram, M. (1995)
Effect of dietary supplementation of different β-carotene isomers on lipoprotein oxidative modification.
J. Nutr. Environ. Med. 5: 13-22.

91. Ben-Amotz, A. (1995)
Derivation of β-carotene from marine organisms.
In: Coastal Ocean Space Utilization. N. Della Croce, S. Connell and R. Abel (eds.). E & FN SPON, London, pp. 433-436.

92. Ben-Amotz, A. (1995)
Simultaneous profiling and identification of carotenoids, retinols and tocopherols by high performance liquid chromatography equipped with three-dimensional photodiode-array detection.

J. Liquid Chromatogr. 18: 2813-2825.

93. Ben-Amotz, A. (1996)
The effect of low temperature on the stereoisomer composition of β-carotene in the halotolerant alga *Dunaliella bardawil* (Chlorophyta).
J. Phycol. 32: 272-275.

94. Ben-Amotz, A. and Levy, Y. (1996)
Bioavailability of a natural isomer mixture compared with synthetic all-trans β-carotene in human serum.
Am. J. Clin. Nutr. 63: 729-734.

95. Levy, Y., Kaplan, M., Ben-Amotz, A. and Aviram, M. (1996)
Effect of dietary supplementation of β-carotene on human monocyte-macrophage-mediated oxidation of low density lipoprotein.
Isr. J. Med. Sci. 32: 473-478.

96. Ben-Amotz, A., Rachmilevich, B., Greenberg, S., Sela, M. and Weshler, Z. (1996)
Natural beta-carotene and whole body irradiation.
Radiat. Environ. Biophys., 35: 285-288.

97. Pipek, R., Dankner, G., Ben-Amotz, A., Aviram, M. and Levy Y. (1996)
Increased plasma oxidizability in subjects with severe obesity. J. Nutr. Environm. Med.,
6: 267-272.

98. Ben-Amotz, A. (1998)
Case study: *Dunaliella* algal derived β-carotene.
In: Phytochemicals: A New Paradigm (Ed. W.R. Bidlack). Technomic Publishing Co., Inc. Lancaster, Basel, pp. 113-125.

99. Ben-Amotz, A. and Fishler R. (1998)
Analysis of carotenoids with emphasis on 9-cis β-carotene in vegetables and fruits commonly consumed in Israel.
Food Chemistry, 62: 515-520.

100. Ben-Amotz, A., Yatziv, S., Sela, M., Greenberg, S., Rachmilevich, B., Shwarzman, M., and Weshler, Z. (1998)
Effect of Natural β-Carotene Supplementation on Children Exposed to Radiation During the Chernobyl Accident.
Radiation & Environmental Biophysics, 37: 187-193.

101. Levy, Y., Paster, E., Dankner, G., Ben-Amotz, A. and Brook J. G. (1998)
Effect of alpha-alpha esterol on plasma cholesterol and antioxidants.
Harefuah, 134: 502-503.

102. Levy, Y., Bartha, P., Ben-Amotz, A., Brook, J.G., et al. (1998)
Plasma antioxidant and lipid peroxidation in acute myocardial infraction and thrombolysis.
J. Am. Coll. Nutr., 17: 337-341.

103. Ben-Amotz, A. (1999)
Production of β-carotene from *Dunaliella*.
In: Microalgae as a source of valuable chemicals, (Ed. Z. Cohen). Taylor & Francis Publishers. London, pp. 196-204.

Levy, Y., Rozen, G. S., Marmor, E., Ben-Amotz, A. and Moshe R. (1999)
Antioxidant vitamin intake in adolescent Israeli girls.
J. Nut. Environm. Med., 9: 23-27.

105. Neuman, I., Nahum, H. and Ben-Amotz A. (1999)
Prevention of exercise-induced asthma by a natural mixture of β-carotene.
Annals of Allergy, Asthma, & Immunology, 82: 549-553.

106. Levy, Y., Zaltsberg, H., Ben-Amotz, A., Kanter Y., and Aviram, M. (1999)
β-Carotene affects antioxidant status in non-insulin-dependent diabetes mellitus
Pathophysiolgy, 6: 157-161.

107. Werman, M.J., Ben-Amotz, A., and Mokady, S. (1999)
Availability and antiperoxidative effects of β-carotene from *Dunaliella bardawil* in alcohol-drinking rats.
J. Nutr. Biochem. 10: 449-454.

108. Ben-Amotz, A. (1999)
Dunaliella β-carotene from science to commerce.
In: Enigmatic Microorganisms and Life in Extreme Environments,
(Ed. J. Seckbach). Kluwer Academic Press, Amsterdam, pp. 399-410.

109. Neuman, I., Nahum, H. and Ben-Amotz A. (2000)
Reduction of exercise-induced asthma oxidative stress by lycopene a natural antioxidant.
Eur. J. Allergy Clin Immunol. 55: 1184-1189.

110. Levy, Y., Zaltsberg, H., Ben-Amotz, A., Kanter Y., and Aviram, M (2000)
Dietary supplementation of a natural isomer mixture of beta-carotene inhibits oxidation of LDL derived from patients with diabetes mellitus.
Annals Nutr. Metab. 44: 54-60.

111. Ben-Amotz, A. (2000)
Nutraceutical use of the microalga *Dunaliella bardawil* against the detrimental effect of irradiation. Proceeding of the Pre-Congress Internet Conference May 8- December 31, 2000. 11th World Congress of Food Science and Technology.

112. Katzor, C., Madar, R., Ben-Amotz, A., and Levy Y. (2002)
Plasma antioxidant vitamins and rheumatic arthritis.
Harefua, 141: 148-150.

113. Reitman, A., Friedrich, I., Ben-Amotz, A., and Levy Y. (2002)
Low plasma antioxidants and normal plasma B vitamins and homocysteine in patients with severe obesity.
IMAJ, 4: 590-593.

114. Werman, M., Mokady, S., and Ben-Amotz A. (2002)
Bioavailability of the isomer mixture of phytoene and phytofluene-rich *Dunaliella bardawil* in rat plasma and tissue. J. Nutr. Biochem. 13: 581-587.

115. Ben-Amotz, A. (2003)
Industrial Production of Microalgal Cell-mass and Secondary Products – Major Industrial Species. In Handbook of Microalgal Cultures, Biotechnology and Applied Phycology. Blackwell, UK.

116. Blum, S., Aviram, M., Zinder, O., Ben-Amotz, A. and Levy Y. (2003)
Mediterranean diet increases postprandial serum paraoxonase activity and decreases serum CRP levels.
Metabolism, in press.

117. Lavy, A., Karban, A., Suissa, A., Yassin, K., Hermesh, I. And Ben-Amotz, A. (2004) Natural b-carotene for the prevention of post-ERCP pancreatitis. Pancreas 29: 45-50.

Annex A

List of Publication

Journal articles

1. **Shaish**, N. Roth-Bejerano and C. Itai **(1989)** The response of stomata to CO_2 relates to its effect on respiration and ATP level. Physiol. Plant. 76:107-111.

2. Ben-Amotz, **A. Shaish** and M. Avron **(1989)** Mode of action of the massively accumulated β-carotene of *Dunaliella bardawil* in protecting the alga against damage by excess irradiation. Plant. Physiol. 91:1041-1043.

3. **Shaish**, M. Avron and A. Ben-Amotz **(1990)** Effect of inhibitors on the formation of stereoisomers in the biosynthesis of β-carotene in *Dunaliella bardawil*. Plant Cell Physiol. 31:689-696.

4. Ben-Amotz, **A. Shaish** and M. Avron **(1991)** The biotechnology of cultivating *Dunaliella* for the production of β-carotene rich algae. Bioresource Technol. 38:233-235.

5. **Shaish**, A. Ben-Amotz and M. Avron **(1991)** Production and selection of high β-carotene mutants of *Dunaliella bardawil*. J. Phycol. 27:652-656.

6. **Shaish**, A. Ben-Amotz and M. Avron **(1992)** Biosynthesis of β-carotene in *Dunaliella*. Methods Enzymol. 213:439-444.

7. **Shaish**, M. Avron, U. Pick and A. Ben-Amotz. **(1993)** Are active oxygen species involved in induction of β-carotene accumulation in *Dunaliella bardawil*. Planta 190:363-368.

8. H. Levi, T. Tal, **A. Shaish** and A. Zamir **(1993)** Cbr, an algal homolog of plant early light-induced proteins, is a putative zeaxanthin binding protein. JBC 268:20892-20896.

9. **Shaish**, **A**. Daugherty, F. O'Sullivan, G. Schonfeld, J. Heinecke. **(1995)** Beta-carotene inhibits atherosclerosis in hypercholesterolemic rabbits. J. Clin. Invest. 96:2075-2082.

10. M. Zelazny, **A. Shaish**, U. Pick. **(1995)** Plasma membrane sterols are essential for sensing osmotic changes in the halotolerant alga *Dunaliella*. Plant. Physiol.109:1395-1403.

11. X. Zhu, D. Noto, R. Seip, **A. Shaish**, A. Daugherty, and G. Schonfeld. **(1997)** Organ loci of catabolism of short truncations of ApoB. Arterioscler. Thromb. Vasc. Biol. 17:1032-1038.

12. **Shaish**, M. Pape, T. Rea, R.K. A. Srivastava, M. Latour, and G. Schonfeld. **(1997)** Alcohol increases diet-induced atherogenic lipoproteins and atherosclerosis lesions in rabbits. Arterioscler. Thromb. Vasc. Biol. 17:1091-1097.

13. Leeuwenburgh, P. Hansen, N., **A. Shaish**, J.O. Holloszy, J.W. Heinecke. **(1998)** Markers of protein oxidation by hydroxyl radical and reactive nitrogen species in tissues of aging rats. Am. J. Physiol. 274:453-461.

14. J. George A. Afek, B. Gilburd, H. Levkovitz, **A. Shaish**, I. Goldberg, Y. Kopolovic, G. Wick, Y. Shoenfeld, D. Harats **(1998)** Hyperimmunization of Apo-E-deficient mice with homologous malondialdehyde low density lipoprotein (MDA-LDL) suppresses early atherogenesis. Atherosclerosis 138:147-152.

15. J. George, A. Afek, B. Gilburd, M. Blank, Y.Levy, A. Aron-Maor, H. Levkovitz, **A. Shaish**, J. Kopolovic, I. Goldberg, D. Harats, Y. Shoenfeld. **(1998)** Induction of early atherosclerosis in LDL-receptor deficient mice immunized with beta 2 glycoprotein I. Circulation 98:1108-1115.

16. J. George, Y. Shoenfeld, A. Afek, B. Gilburd, P. Keren, **A. Shaish**, J. Kopolovitc, D. Harats. **(1999)** Enhanced fatty streak formation in C57BL/6 mice by immunization with heat-shock-protein 65. Arterioscler. Thromb. Vasc. Biol. 19:505-510.

17. Afek, J. George, Y. Shoenfeld, B. Gilburd, Y. Levi, **A. Shaish**, P. Keren, I. Goldberg, J. Kopolovitc, D. Harats. **(1999)** Enhancement of atherosclerosis in beta 2 glycoprotein I immunized apo-E deficient mice. Pathobiology 67:19-25.

18. **Shaish** J. George, H. Levkovitz, , B. Gilburd, P. Keren, D. Harats. **(1999)** Dietary β-carotene and α-tocopherol combination does not inhibit atherogenesis in an apoE-deficient mouse model. Arterioscler. Thromb. Vasc. Biol. 19:1470-1475.

19. Y. Sherer, **Shaish A**, H. Levkovitz, Y. Shoenfeld, D. Harats. **(1999)** Magnesium fortification of drinking water suppresses atherogenesis in male LDL-receptor knockout mice. Pathobiology 67:207-213.

20. P. Keren, J. George, **A. Shaish**, H. Levkovitz, Z. Janakovic,A. Afek, I. Goldberg, J. kopolovic, G. Keren, D. Harats. **(2000)** Effect of hyperglycemia and hyperlipidemia on atherosclerosis in LDL-receptor deficient mice: establishement of a combined model and association with heat shock protein65 immunity. Diabetes 49:1064-1069.

21. Y. Sherer, Y. Shoenfeld, **A. Shaish**, H. Levkovitz, R. Bitzur, D. Harats. **(2000)** Suppression of atherosclerosis in female low-density lipoprotein receptor knockout mice following magnesium fortification of drinking water: the importance of diet. Pathobiology 68:93-98.

22. D. Harats, **A. Shaish**, J. George, M. Mulkins, H. Kurihara, H. Levkovitz E.. Sigal,. **(2000)** Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. Arterioscler. Thromb. Vasc. Biol. 20:2100-2105.

23. J. George, D. Harats, B. Gilburd, A. Afek, **A. Shaish,** J. Kopolovitc, Y. Shoenfeld. **(2000)** Adoptive transfer of beta(2)-glycoprotein I-reactive lymphocytes enhances early atherosclerosis in LDL receptor-deficient mice. Circulation 102:1822-1827. 24.

24. George J, Shoenfeld Y, Gilburd B, Afek A, **Shaish A**, Harats D. **(2000)** Requisite role for interleukin-4 in the acceleration of fatty streaks induced by heat shock protein 65 or Mycobacterium tuberculesis. Cir. Res. 86:1203-1210.

25. George J, Mulkins M, **Shaish A**, Casey S, Schatzman R, Sigal E, Harats D. **(2000)** Interleukin (IL)-4 deficiency does not influence fatty streak formation in C57BL/6 mice. Atherosclerosis 153:403-411.

26. Sharabi Y, Grossman E, Sherer Y, **Shaish A**, Levkovitz H, Bitzur R, D. Harats. **(2000)** The effect of renin-angiotensin axis inhibition on early atherosclerosis in LDL-receptor-deficient mice. Pathobiology 68:270-274.

27. Varda -Bloom N. **A. Shaish**, A. Gonen, K. Levanon, S. Greenberger, S. Ferber, H. Levkovitz, D. Castel, I. Goldberg, A. Afek, Y. Kopolovitc, D. Harats. **(2001)** Tissue-specific gene therapy directed to tumor angiogenesis. Gene Therapy 8:819-827.

28. D. Harats, O. Yodfat, R. Doolman, S. Gaumendo, D. Marko, **A. Shaish**, S. Ben-Ami. **(2001)** Homocysteine elevation with fibrates: is it a class effect? Isr Med Assoc J 3:243-246.

29. Dar P, Strassburger D, **Shaish A**, Levkovitz H, Halperin R, Harats D. **(2001)** Reduced reproduction with increased abortion rate in transgenic mice that overexpress 15-lipoxygenase. Gynecologic and Obstetric Investigation. 52:18-21.

30. Y. Sherer, R. Bitzur, H. Cohen, **A. Shaish**, D. Varon, Y. Shoenfeld, D. Harats. **(2001)** Mechanism of action of the anti-atherogenic effect of magnesium: lessons from a mouse model. Magnesium Research 14:173-179.

31. George J, Afek A, **Shaish A**, Levkovitz H, Bloom N, Cyrus T, Zhau L, Funk CD, Sigal E, Harats D. **(2001)** 12/15-Lipoxygenase gene disruption attenuates atherosclerosis in LDL receptor-deficient mice. Circulation 104:1646-1650.

32. **A. Shaish**, G. Keren, P. Chouraqui, H. Levkovitz, D. Harats. **(2001)** Imaging of the aortic atherosclerotic lesions by ^{125}I-LDL, ^{125}I-oxidized-LDL, ^{125}I-HDL and ^{125}I-BSA. Pathobiology 69:225-229.

33. Hofit Cohen, Yariv Sherer, **Aviv Shaish**, Yehuda Shoenfeld, Hana Levkovitz, Refael Bizur, Dror Harats. **(2002)** Atherogenesis inhibition induced by magnesium-chloride fortification of drinking water. Biological Trace Element Research 1-3:251-269.

34. Zohar Levy, **Aviv Shaish**, Niva Yacov, Hana Levkovitz, Svetlana Trestman, Yariv Gerber, Hofit Cohen, Ayana Dvir, Rita Rhachamani and Dror Harats. **(2003)** Rosiglitazone (PPARγ-agonist) attenuates atherosclerosis with no effect on hyperglycemia in a combined diabetes-atherosclerosis mouse model. Diabetes, Obesity and Metabolism 5:1-6.

35. Gonen A, **Shaish A**, Leikin-Frenkel A, Gilat T, Dror Harats. **(2003)** The fatty acid bile acid conjugates (FABACs) inhibits atherosclerosis in C57BL/6 model. Pathobiology 70:215-218.

36. Levy Z, Dvir A, **Shaish A**, Trestman S, Cohen H, Levkovietz H, Rhachmani R, Ravid M, Harats D. **(2003)** Omapatrilat, an Angiotensin-Converting Enzyme and Neutral Endopeptidase Inhibitor, Attenuates Early Atherosclerosis in Diabetic and in Nondiabetic Low-Density Lipoprotein Receptor-Deficient Mice. Experimental Diab. Res. 4:59-64.

37. Levy Z, Rachmani R, Trestman S, Dvir A, **Shaish A**, Ravid M, Harats D. Low-dose interferon-alpha accelerates atherosclerosis in an LDL receptor-deficient mouse model.Eur J Intern Med. 2003 Dec;14(8):479-483

38. Shoshana Greenberger, Nira Varda-Bloom, Keren Levanon, Eyal Breitbart, Iris Goldberg, Iris Barshack, Israel Hodish, Niva Yaacov, Livnat Bangio, Tanya Goncharov, David Wallach, **Aviv Shaish**, Dror Harats. **(2004)** Tanscription-controlled gene therapy against tumor angiogenesis. J. Clin. Invest. J Clin Invest. 2004 Apr;113(7):1017-24.

39. Jacob George, Niva Yacov, Eyal Breitbart, **Aviv Shaish**, Boris Gilburd, Yehuda Shoenfeld, Dror Harats. **(2004)** Suppression of Early Atherosclerosis in LDL-Receptor Deficient Mice by Oral Tolerance with β2-Glycoprotein I. Cardiovasc Res. 2004 Jun 1;62(3):603-9.

Chapters in Books

1. A. Ben-Amotz and **A. Shaish. (1992)** β-carotene synthesis. In: *Dunaliella*: Physiology, Biochemistry and Biotechnology (A. Ben-Amotz ed.) CRC Press Inc. pp.206-216.

2. Sherer, Y., Blank, M., Varrala, O., **Shaish, A.**, Shoenfeld, Y., Harats, D. Anti-prothrombin antibodies in thrombosis and atherosclerosis. In: Atherosclerosis and Autoimmunity; Elsevier, 185-190, 2001.

Abstracts and Proceedings

1. C. Itai, A. Nejidat, **A. Shaish** and N. Roth-Bejerano **(1988)** Phytochrome involvement in stomatal movement pp 95-101. In: Time scales and water stress-Proc. 5th Int. Conf. on Mediterranean Ecosystem. Di. Castri F., Floret Ch., Rambais S., Roy J. (Eds.) I.U.B.S Paris.

2. **Shaish A.** M. Avron and A. Ben-Amotz. **(1990)** Effect of inhibitors on the formation of stereoisomers in the biosynthesis of β-carotene in *Dunaliella bardawil.* 9th International Symposium on Carotenoids. Kyoto, Japan.

3. **Shaish A,** G. Schonfeld and J.W. Heinecke **(1994)** All trans and 9-cis β-carotene inhibit LDL and HDL oxidation by aqueous peroxyl radical. Circulation 90:I-409.

4. R.A.K. Srivastava, N. Srivastava, **A. Shaish**, G. Schonfeld. **(1995)** Cholic acid lowers plasma apoAI levels in non-transgenic and in apoAI transgenic mice at posttranslational loci of regulation. Circulation Supplement 92:1672.

5. D. Harats, J. George, A. Afek, B. Gilburd, H. Levkovitz, **A. Shaish**, I. Goldberg, Y. Kopolovic, Y. Shoenfeld. **(1997)** Hyperimmunization of chow-fed ApoE deficient mice with homologous oxidized low-density lipoprotein suppresses early atherosclerosis. Atherosclerosis 134:18.

6. **Shaish A.**, J. George, A. Afek, B. Gilburd, H. Levkovitz, I. Goldberg, Y. Kopolovic, Y. Shoenfeld, D. Harats. **(1997)** Induction of early atherosclerosis in LDL-receptor deficient mice immunized with Beta 2 glycoprotein I. Atherosclerosis 134:39.

7. D. Harats, H. Kurihara, H. Levkovitz, **A. Shaish**, E. Sigal. **(1997)** 15-lipoxygenase overexpression induces atherosclerosis in LDL deficient mice. Atherosclerosis 134:279.

8. Harats, D, Kurihara, H., **Shaish, A.**, Sigal, E. **(1997)** 15-Lipoxygenase overexpression induces atherosclerosis in LDL -receptor deficient mice. Circulation, (96) 8: I 167

9. Harats, D., George, J., Afec, A., Gillburd, B., Levkovitz, H., **Shaish, A.**, Goldberg, I., Kopolovitz, Y., Shoenfeld, Y. **(1997)** Hyperimmunization of chow fed ApoE defficient mice with homologous oxidized Low Density Lipoprotein suppresses early atherogenesis. Circulation 134:18.

10. Harats, D., **Shaish, A.**, George, J., Afec, A., Gillburd, B., Kopolovitz,Y., Shoenfeld, Y. **(1997)** Induction of early atherosclerosis in LDL-receptor deficient mice immunized with beta 2 GPI. Circulation 134:39-40.

11. Harats, D, **Shaish**, A., Levkovitz., Sigal, E., Bloom, VN. **(1998)** Tissue specific gene therapy to the vascular wall. In the proceedings of the Keystone Symposia on Gene-Therapy Keystone, USA.

12. Harats, D., George, J., Afek, A., Gillburd, B., Levkovitz, H., **Shaish, A.**, Kopolovitz, Y., Wick, G., Shoenfeld, Y. **(1998)** Immunomodulation of atherogenesis. In the proceedings of the 70th European Atherosclerosis society, Geneva, Switzerland, 61.

13. **Shaish, A.**, Levkovitz, H., George, J., Gillburd, B., Keren, P., Harats, D. **(1998)** Antioxidant vitamins do not inhibit atherogenesis in an Apolipoprotein E-deficient mouse model. In the proceedings of the 70th European Atherosclerosis society, Geneva, Switzerland, 34,

14. George, J., Shoenfeld, Y., Afek, A., Gillburd, B., Keren, P., **Shaish, A.**, Kopolovitz, Y., Wick, G., and Harats, D. **(1998)** "Enhanced fatty streak formation in C57BL/6 mice by immunization with heat-shock protein 65" In the proceedings of the 70th European Atherosclerosis society, Geneva, Switzerland, 131.

15. Bloom, VN., **Shaish, A.**, Levkovitz., Sigal, E., Harats, D. **(1998)** Tissue specific gene therapy to the vascular wall. In the proceedings of the Scientific Meeting of the Society for Research, Prevention and Treatment of Atherosclerosis, Ashkelon, Israel.

16. George, J., Shoenfeld, Y., Afek, A., Gillburd, B., Keren, P., **Shaish, A.**, Kopolovitz, Y., Wick, G., and Harats, D. **(1998)** "Enhanced fatty streak formation in C57BL/6 mice by immunization with heat-shock protein 65" In the proceedings of the Scientific Meeting of the Society for Research, Prevention and Treatment of Atherosclerosis, Ashkelon, Israel.

17. **Shaish, A.**, George, J., Gillburd, B., Keren, P., Levkovitz, H., Harats, D. **(1998)** Dietary B-carotene and alpha-tocopherol combination dose not inhibit atherogenesis in an Apolipoprotein E-deficient mouse model. In the proceedings of the Scientific Meeting of the Society for Research, Prevention and Treatment of Atherosclerosis, Ashkelon, Israel.

18. **Shaish A**, Y. Sherer, H. Levkovitz, Y. Shoenfeld, D. Harats. **(1999)** Magnesium fortification of drinking water suppresses atherogenesis in male LDL-receptor knockout mice. Atherosclerosis 144:96.

19. Keren P., J. George, **A. Shaish**, H. Levkovitz, B. Gilburd, Z. Janackovic, G. Green, Y. Shoenfeld, D. Harats. **(1999)** Combined hyperlipidemic hyperglycemic model of atherosclerosis in apoE deficient mice. Atherosclerosis 144:97.

20. D. Harats, J. George, B. Gilburd, A. Afek, **A. Shaish**, Y. Shoenfeld. **(1999)** β2-glucoprotein I (apolipoprotein-H) as an influential determinant in atherosclerosis. Atherosclerosis 144:122.

ANNEX B

The New England Journal of Medicine

VITAMIN E SUPPLEMENTATION AND CARDIOVASCULAR EVENTS IN HIGH-RISK PATIENTS

THE HEART OUTCOMES PREVENTION EVALUATION STUDY INVESTIGATORS*

ABSTRACT

Background Observational and experimental studies suggest that the amount of vitamin E ingested in food and in supplements is associated with a lower risk of coronary heart disease and atherosclerosis.

Methods We enrolled a total of 2545 women and 6996 men 55 years of age or older who were at high risk for cardiovascular events because they had cardiovascular disease or diabetes in addition to one other risk factor. These patients were randomly assigned according to a two-by-two factorial design to receive either 400 IU of vitamin E daily from natural sources or matching placebo and either an angiotensin-converting–enzyme inhibitor (ramipril) or matching placebo for a mean of 4.5 years (the results of the comparison of ramipril and placebo are reported in a companion article). The primary outcome was a composite of myocardial infarction, stroke, and death from cardiovascular causes. The secondary outcomes included unstable angina, congestive heart failure, revascularization or amputation, death from any cause, complications of diabetes, and cancer.

Results A total of 772 of the 4761 patients assigned to vitamin E (16.2 percent) and 739 of the 4780 assigned to placebo (15.5 percent) had a primary outcome event (relative risk, 1.05; 95 percent confidence interval, 0.95 to 1.16; P=0.33). There were no significant differences in the numbers of deaths from cardiovascular causes (342 of those assigned to vitamin E vs. 328 of those assigned to placebo; relative risk, 1.05; 95 percent confidence interval, 0.90 to 1.22), myocardial infarction (532 vs. 524; relative risk, 1.02; 95 percent confidence interval, 0.90 to 1.15), or stroke (209 vs. 180; relative risk, 1.17; 95 percent confidence interval, 0.95 to 1.42). There were also no significant differences in the incidence of secondary cardiovascular outcomes or in death from any cause. There were no significant adverse effects of vitamin E.

Conclusions In patients at high risk for cardiovascular events, treatment with vitamin E for a mean of 4.5 years has no apparent effect on cardiovascular outcomes. (N Engl J Med 2000;342:154-60.)

OXIDATIVE modification of low-density lipoprotein is an important step in the development and progression of atherosclerosis in experimental studies,[1,2] and antioxidants such as vitamin E have been shown to slow atherosclerosis.[3-5] An inverse relation has been observed between coronary heart disease and the consumption of fruits, vegetables, and other foods containing vitamins, particularly vitamin E.[6-9] Observational studies have indicated that persons who consume more than 100 IU of vitamin E a day for more than two years have lower rates of coronary events[10,11] and lower rates of progression of coronary artery lesions.[12] However, observational studies cannot distinguish whether the lower risk of coronary heart disease associated with higher levels of vitamin E consumption is due to the vitamin or to other associated lifestyle factors such as increased exercise and other aspects of diet. There have been four randomized, controlled trials of the relation between vitamin E and coronary heart disease,[13-16] but their results are conflicting, perhaps because of the low doses of vitamin E used in some studies,[13,14] the small numbers of events,[15] or the limited duration of treatment.[15,16]

We evaluated a high dose (400 IU per day) of vitamin E from natural sources, which has high bioavailability, in a large, five-year, prospective study of patients at high risk for cardiovascular events. The primary outcome was a composite of myocardial infarction, stroke, and death from cardiovascular causes. The secondary outcomes included death from any cause, hospitalization for unstable angina or congestive heart failure, revascularization or limb amputation, complications of diabetes, and cancer. The trial was also designed to evaluate the effects of an angiotensin-converting–enzyme inhibitor, ramipril, on the incidence of cardiovascular events. After nearly 4.5 years of follow-up, the collection of data on cardiovascular disease was stopped in April 1999 on the basis of a finding by the independent data and safety monitoring board that the trial had conclusively demonstrated the benefits of ramipril and a lack of effect of vitamin E on cardiovascular events. This report presents our findings relating to the effects of vitamin E on the primary and secondary cardiovascular outcomes. The study has been continued in the majority of centers to evaluate the effects of vitamin E on the incidence of cancer.

METHODS

Study Design

The Heart Outcomes Prevention Evaluation (HOPE) Study is a double-blind, randomized trial with a two-by-two factorial de-

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The writing group (Salim Yusuf, D.Phil., Gilles Dagenais, M.D., Janice Pogue, M.Sc., Jackie Bosch, M.Sc., and Peter Sleight, D.M.) assumes responsibility for the overall content and integrity of the manuscript.

*The investigators are listed in the Appendix of the Heart Outcomes Prevention Evaluation Study Investigators. Effects of an Angiotensin-Converting–Enzyme Inhibitor, Ramipril, on Cardiovascular Events in High-Risk Patients. N Engl J Med 2000;342:145-53.

sign, conducted to evaluate the effects of ramipril and vitamin E in 9541 patients at high risk for cardiovascular events. The results of the comparison of ramipril with placebo are reported in a companion article.[17] Details of the methods are given in that article[17] and in a previously published article.[18] Briefly, eligible patients at high risk were randomly assigned to receive either 400 IU of vitamin E from natural sources or an equivalent placebo daily for 4 to 6 years (mean, 4.5) and in addition to receive either 10 mg of ramipril or an equivalent placebo daily. Patients were evaluated every six months for a variety of outcomes.

Outcomes

The primary outcome was a composite of myocardial infarction, stroke, and death from cardiovascular causes. Deaths classified as due to cardiovascular causes were unexpected deaths presumed to be due to ischemic cardiovascular disease and occurring within 24 hours after the onset of symptoms without clinical or postmortem evidence of another cause; deaths from myocardial infarction or stroke that occurred within seven days after the myocardial infarction or stroke; and deaths from congestive heart failure, dysrhythmia, pulmonary embolism, or ruptured abdominal aortic aneurysm. Deaths for which the cause was uncertain were presumed to be due to cardiovascular disease. Myocardial infarction was diagnosed when two of the following three criteria were met: typical symptoms, increased cardiac enzyme levels (at least twice the upper limit of normal), and diagnostic electrocardiographic changes. Stroke was defined as a neurologic deficit lasting more than 24 hours. A computed tomographic or magnetic resonance imaging examination was recommended to define the type of stroke.

Secondary and other outcomes were death from any cause; unstable angina, defined as worsening angina or angina at rest requiring hospitalization; hospitalization for heart failure with clinical and radiologic signs of congestion; revascularization or limb amputation; the development of overt nephropathy or the need for dialysis or laser therapy among patients with diabetes; and the development of heart failure or new or worsening angina regardless of the need for hospitalization.

RESULTS

Characteristics of the Patients

The characteristics of the 9541 patients are shown in Table 1. The rate of compliance with the assigned regimen was high throughout the study. The percentages of patients who were taking vitamin E in the vitamin E and placebo groups, respectively, were 94.2 percent and 1.0 percent at one year, 93.3 percent and 1.7 percent at two years, 91.3 percent and 2.0 percent at three years, 90.2 percent and 2.7 percent at four years, and 89.2 percent and 3.4 percent at the final visit.

TABLE 1. BASE-LINE CHARACTERISTICS OF THE PATIENTS.*

CHARACTERISTIC†	VITAMIN E GROUP (N=4761)	PLACEBO GROUP (N=4780)
Age — yr	66±7	66±7
Blood pressure — mm Hg	139±20/79±11	139±20/79±11
Heart rate — beats/min	69±11	69±11
Body-mass index‡	28±4	28±4
Female sex — no. (%)	1263 (26.5)	1282 (26.8)
History of coronary artery disease — no. (%)	3857 (81.0)	3832 (80.2)
Myocardial infarction	2499 (52.5)	2535 (53.0)
Stable angina pectoris	2653 (55.7)	2668 (55.8)
Unstable angina pectoris	1205 (25.3)	1246 (26.1)
CABG	1229 (25.8)	1251 (26.2)
PTCA	851 (17.9)	863 (18.1)
Stroke or transient ischemic attacks — no. (%)	530 (11.1)	500 (10.5)
Peripheral vascular disease — no. (%)§	2109 (44.3)	2037 (42.6)
Hypertension — no. (%)	2219 (46.6)	2222 (46.5)
Diabetes — no. (%)	1838 (38.6)	1816 (38.0)
Known elevated total cholesterol — no. (%)	3109 (65.3)	3171 (66.3)
Known low HDL cholesterol — no. (%)	893 (18.8)	869 (18.2)
Current cigarette smoking — no. (%)	665 (14.0)	679 (14.2)
Medications — no. (%)		
Beta-blockers	1901 (39.9)	1870 (39.1)
Aspirin or other antiplatelet agents	3665 (77.0)	3616 (75.6)
Lipid-lowering agents	1352 (28.4)	1401 (29.3)
Diuretics	728 (15.3)	717 (15.0)
Calcium-channel blockers	2249 (47.2)	2236 (46.8)
Left ventricular hypertrophy on ECG — no. (%)	411 (8.6)	382 (8.0)
Microalbuminuria — no. (%)	1012 (21.3)	976 (20.4)

*Plus–minus values are means ±SD.

†CABG denotes coronary-artery bypass grafting, PTCA percutaneous transluminal coronary angioplasty, HDL high-density lipoprotein, and ECG electrocardiogram.

‡The body-mass index is calculated as the weight in kilograms divided by the square of the height in meters.

§Peripheral vascular disease included claudication, a history of peripheral arterial disease, or a ratio of blood pressure in the ankle to blood pressure in the arm of less than 0.90.

Primary Cardiovascular Outcomes and Deaths from Any Cause

A total of 772 of the 4761 patients who were assigned to receive vitamin E (16.2 percent) and 739 of the 4780 who were assigned to placebo (15.5 percent) had a primary cardiovascular event (relative risk, 1.05; 95 percent confidence interval, 0.95 to 1.16; P=0.33) (Table 2 and Fig. 1). There were no significant differences between the groups in the numbers of deaths from cardiovascular causes (342 in the vitamin E group vs. 328 in the placebo group; relative risk, 1.05), myocardial infarctions (532 vs. 524; relative risk, 1.02), deaths from coronary heart disease (287 vs. 277; relative risk, 1.06), or strokes (209 vs. 180; relative risk, 1.17) (Fig. 2 and 3). The total numbers of deaths were similar in the two groups (535 vs. 537; relative risk, 1.00). Vitamin E had no significant effect on the primary outcome either among patients who were receiving ramipril (338 events among those who were receiving vitamin E and 313 events among those who were receiving placebo; relative risk, 1.08) or among patients who were not receiving ramipril (421 and 405 events, respectively; relative risk, 1.05).

Secondary Cardiovascular and Combined Outcomes

There were no differences between patients assigned to vitamin E and those assigned to placebo in the number of hospitalizations for unstable angina

TABLE 2. INCIDENCE OF THE PRIMARY OUTCOME AND OF DEATHS FROM ANY CAUSE.

OUTCOME	VITAMIN E GROUP (N=4761)	PLACEBO GROUP (N=4780)	RELATIVE RISK (95% CI)*	P VALUE†
	no. (%)			
Myocardial infarction, stroke, or death from cardiovascular causes‡	772 (16.2)	739 (15.5)	1.05 (0.95–1.16)	0.33
Death from cardiovascular causes§	342 (7.2)	328 (6.9)	1.05 (0.90–1.22)	0.54
Myocardial infarction§	532 (11.2)	524 (11.0)	1.02 (0.90–1.15)	0.74
Stroke§	209 (4.4)	180 (3.8)	1.17 (0.95–1.42)	0.13
Death from any cause	535 (11.2)	537 (11.2)	1.00 (0.89–1.13)	0.99

*CI denotes confidence interval.

†P values were calculated with use of the log-rank test.

‡The number of events among those receiving ramipril did not differ significantly between those assigned to receive vitamin E and those assigned to placebo (338 vs. 313). Similar results were observed among those who received matching placebo rather than ramipril (421 vs. 405).

§A patient may have had more than one event.

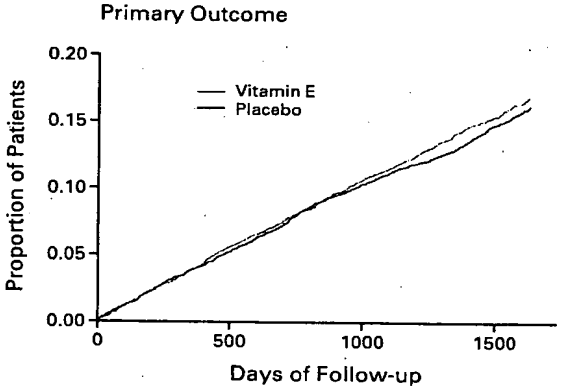

Figure 1. Kaplan–Meier Estimates of the Effect of Vitamin E on the Composite Outcome of Nonfatal Myocardial Infarction, Stroke, or Death from Cardiovascular Causes.

The relative risk of the composite outcome in the vitamin E group as compared with the placebo group was 1.05 (95 percent confidence interval, 0.95 to 1.16; P=0.33).

(586 vs. 569; relative risk, 1.04), hospitalizations for heart failure (160 vs. 144; relative risk, 1.12), or revascularizations or limb amputations (848 vs. 787; relative risk, 1.09) (Table 3). There were no significant differences in the number of patients with angina of new onset (278 vs. 245; relative risk, 1.15) or microvascular complications of diabetes (340 vs. 325; relative risk, 1.06). A combined analysis of the proportion of patients who had any primary or secondary event found a nonsignificantly higher rate among those assigned to vitamin E (1630 vs. 1576; relative risk, 1.05; 95 percent confidence interval, 0.98 to 1.13; P=0.14).

Subgroup Analyses

There was no heterogeneity of results among subgroups defined according to sex, age, previous cardiovascular disease, or use of other drugs with respect to the primary or secondary outcomes (data not shown). Specifically, there was no significant difference in the incidence of the primary outcome among patients with diabetes (325 of those assigned to vitamin E vs. 313 of those assigned to placebo; relative risk, 1.04) or among smokers (135 vs. 139; relative risk, 1.02).

Adverse Effects

There was no significant difference between groups in the incidence of adverse effects or in the number of patients who stopped taking the study medication. There was no increase in hemorrhagic stroke associated with vitamin E use (17 of those assigned to vitamin E had hemorrhagic stroke, as compared with 13 of those assigned to placebo) or among those who were also taking an antiplatelet agent (11 vs. 8).

DISCUSSION

In our study, vitamin E did not reduce the incidence of cardiovascular events, as compared with the incidence among patients assigned to placebo, during a follow-up period of four to six years. Given the large number of events and the consistent lack of difference in all secondary cardiovascular outcomes, it is very unlikely that vitamin E had any clinically worthwhile beneficial effect on cardiovascular disease during four or five years of treatment.

Results have been reported from four randomized trials of the effects of vitamin E on cardiovascular events. In a Chinese study, 29,584 adults from Linxian Province, who did not have cardiovascular disease at entry, were randomly assigned to receive daily vita-

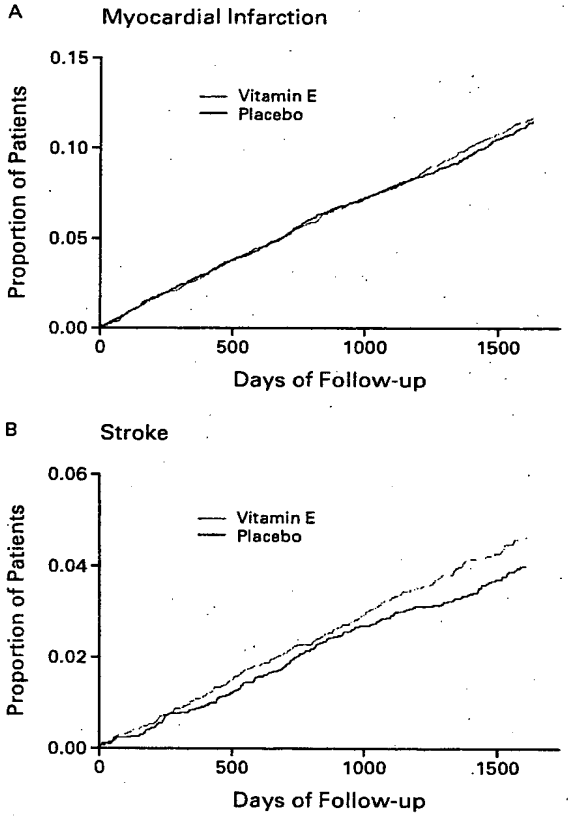

Figure 2. Kaplan–Meier Estimates of the Effect of Vitamin E on the Incidence of Myocardial Infarction (Panel A) and Stroke (Panel B).

The relative risk of myocardial infarction in the vitamin E group as compared with the placebo group was 1.02 (95 percent confidence interval, 0.90 to 1.15; P=0.74), and the relative risk of stroke was 1.17 (95 percent confidence interval, 0.95 to 1.42; P=0.13).

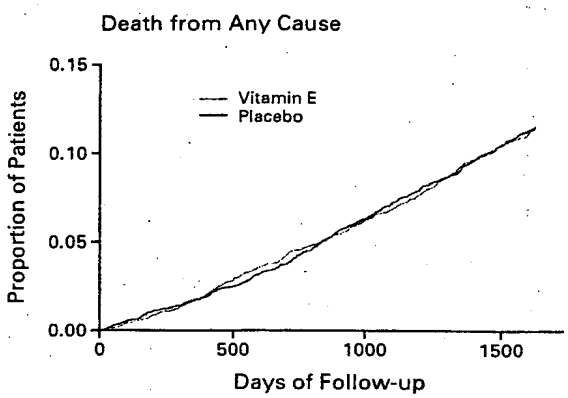

Figure 3. Kaplan–Meier Estimates of the Effect of Vitamin E on the Incidence of Death from Any Cause.

The relative risk in the vitamin E group as compared with the placebo group was 1.00 (95 percent confidence interval, 0.89 to 1.13; P=0.99).

min E (30 mg), beta carotene, and selenium supplements or to receive placebo.[13] During the 5.2 years of follow-up, there was a 9 percent decrease in deaths from any cause without any significant reduction in cardiovascular events. The dose of vitamin E in this study was small, the nutritional status and cardiovascular risk of this population were very different from those of Western populations, and the beneficial effects on overall mortality cannot be attributed only to vitamin E.

The second trial was the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study, involving 29,133 male smokers who were 50 to 69 years of age.[14] Daily treatment with 50 mg of vitamin E for five to eight years had no effect on the risk of death from coronary heart disease. In a subgroup of 1862 men with a previous myocardial infarction at entry, there was a nonsignificant increase in the risk of death from coronary heart disease (relative risk, 1.33; 95 percent confidence interval, 0.86 to 2.05; P=0.20). However, a reduction in the risk of nonfatal myocardial infarction was documented among men assigned to vitamin E only (40 vs. 55; relative risk, 0.62; 95 percent confidence interval, 0.41 to 0.96), but not among those receiving the combination of vitamin E and beta carotene, in comparison with those receiving placebo only.[19] In this subgroup, the number of events was small. In the remaining patients in this study, there was no significant effect of vitamin E on nonfatal or fatal myocardial infarction, despite large numbers of events (1204 and 907, respectively).[20] Thus, in this well-conducted trial, vitamin E had no effect on coronary heart disease. Although the trial used a low dose of synthetic vitamin E (50 mg per day), the median level of alpha-tocopherol increased significantly, from 28.5 μmol per liter at base line to 42.5 μmol per liter at three months.

The third trial was the Cambridge Heart Antioxidant Study, which randomly assigned 2002 patients with coronary atherosclerosis to receive either vitamin E or placebo.[15] The mean alpha-tocopherol levels increased from 34.2 to 51.1 μmol per liter in patients receiving 400 IU of vitamin E per day and to 64.5 μmol per liter in patients receiving 800 IU per day. The majority of the patients received 400 IU per day. After a median follow-up of 1.4 years, a large reduction in the number of patients with nonfatal myocardial infarction was observed (14 in the vitamin E group vs. 41 in the placebo group; relative risk, 0.53; 95 percent confidence interval, 0.11 to 0.47; P=0.005), but there was no difference in deaths due to

TABLE 3. INCIDENCE OF SECONDARY AND OTHER OUTCOMES.

OUTCOME	VITAMIN E GROUP (N=4761)	PLACEBO GROUP (N=4780)	RELATIVE RISK (95% CI)*	P VALUE†
	no. (%)			
Revascularization or limb amputation	848 (17.8)	787 (16.5)	1.09 (0.99–1.20)	0.07
Hospitalization for unstable angina	586 (12.3)	569 (11.9)	1.04 (0.93–1.17)	0.52
New-onset angina	278 (5.8)	245 (5.1)	1.15 (0.97–1.37)	0.11
Worsening angina	1215 (25.5)	1186 (24.8)	1.02 (0.94–1.11)	0.63
Claudication	762 (16.0)	753 (15.8)	1.02 (0.92–1.13)	0.70
Hospitalization for heart failure	160 (3.4)	144 (3.0)	1.12 (0.90–1.41)	0.32
Heart failure	530 (11.0)	457 (9.6)	1.17 (1.03–1.32)	0.02
Complications of diabetes‡	340 (7.1)	325 (6.8)	1.06 (0.91–1.23)	0.47

*CI denotes confidence interval.

†P values were calculated with use of the log-rank test.

‡Complications included nephropathy, dialysis, and laser therapy.

TABLE 4. META-ANALYSIS OF THE EFFECTS OF VITAMIN E ON MYOCARDIAL INFARCTION, STROKE, OR DEATH FROM CARDIOVASCULAR CAUSES IN LARGE TRIALS.*

STUDY	DAILY DOSE	DURATION OF STUDY	VITAMIN E	PLACEBO	RELATIVE RISK (95% CI)
	mg	yr	no. with events/total no. (%)		
ATBC[14]	50	5.0	1889/14,564 (13.0)	1970/14,569 (13.5)	0.96 (0.90–1.03)
CHAOS[15]	≥400	1.3	41/1035 (4.0)	64/967 (6.6)	0.60 (0.40–0.89)
GISSI[16]	300	3.5	571/5660 (10.1)	584/5664 (10.3)	0.98 (0.87–1.10)
Current study	400	4.5	772/4761 (16.2)	739/4780 (15.5)	1.05 (0.95–1.16)
Total			3273/26,020 (12.6)	3357/25,980 (12.9)	0.97 (0.92–1.02)†

*CI denotes confidence interval, ATBC Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, CHAOS Cambridge Heart Antioxidant Study, and GISSI Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico.

†Relative risks and confidence intervals were derived by the method of Yusuf et al.[21]; P=0.27.

cardiovascular causes (27 vs. 23; relative risk, 1.18; 95 percent confidence interval, 0.62 to 2.27; P=0.61). In this trial, the number of events was small and there were imbalances in several base-line characteristics that call into question whether randomization resulted in truly comparable groups.

Furthermore, the very large reduction in nonfatal myocardial infarction within a relatively short time (median, 1.4 years) is inconsistent with the results of other interventions, such as lipid-lowering agents or antihypertensive medications, that reduce cardiovascular events. It is therefore likely that the results of the Cambridge Heart Antioxidant Study may have been due to chance. This possibility is supported by the results of a recent Italian trial,[16] in which 11,000 patients who had had myocardial infarctions were randomly assigned to receive 300 IU of vitamin E per day or placebo for a median of 3.5 years. The number of patients with nonfatal myocardial infarction was slightly higher in the vitamin E group than the placebo group (295 vs. 284; relative risk, 1.02; 95 percent confidence interval, 0.87 to 1.21), and the number of deaths from coronary heart disease was slightly smaller (227 vs. 249; relative risk, 0.92; 95 percent confidence interval, 0.77 to 1.11). Neither difference was statistically significant.[16]

Our study used a high dose of vitamin E (400 IU per day), had high rates of compliance, and involved high-risk patients. The study had a large number of primary outcomes and therefore had high statistical power (more than 90 percent power to detect a 13 percent relative reduction in the risk of the primary outcome). Furthermore, a large number of secondary outcomes (e.g., revascularization or limb amputation, unstable angina, worsening angina, and heart failure) were examined. Such data are not available from most trials. Combining the data from all trials of vitamin E indicates that such treatment has little effect on the risk of death or cardiovascular events (Table 4), at least over a four-to-six-year period.

Steinberg has hypothesized that unlike agents that lower cholesterol or blood pressure, antioxidants may have to be used for more than five years to have a demonstrable benefit, since the primary mechanism of these agents may be the prevention of new lesions.[22] Therefore, in a population like the one we studied, it may take longer than five years to detect an effect on clinical outcomes. However, the Physicians' Health Study did not find a benefit of beta carotene (another antioxidant with a different action) after 12 years.[23] Similar data are not available for vitamin E, but observational studies that demonstrated a lower rate of coronary heart disease with vitamin E supplementation suggested that a lower risk should be evident after two years.[10,11] In a nested substudy, we are examining whether the thickness of the carotid intima and media (an indication of the risk of early atherosclerosis) can be favorably altered by vitamin E.[24] If so, Steinberg's hypothesis may be worth exploring with more prolonged follow-up or treatment to assess whether such changes in the development of atherosclerosis would translate into a benefit in terms of clinical outcomes.

Although the moderate duration of vitamin E supplementation (four to six years) and the characteristics of the population may explain our finding of a lack of benefit of vitamin E, another reason may be our use of vitamin E alone, without other antioxidants. In the epidemiologic studies that found an association between higher dietary intake of vitamin E and lower rates of coronary heart disease, higher vitamin E consumption was also associated with higher intake of a number of other antioxidants and micronutrients.[6-9] It is possible that vitamin E supplementation requires these cofactors to have a beneficial effect.[25] Although the existence of interactions between vitamin E and other vitamins,[10] beta carotene,[6,14] or selenium[13] is not supported by the findings of prospective observational studies or randomized trials, this hypothesis can be tested only in trials in which combinations of vitamins are given; some such trials are now in progress.[26-28]

In conclusion, 400 IU of vitamin E administered daily for four to six years had no beneficial effects on cardiovascular outcomes in a high-risk population of patients who were 55 years of age or older. Vitamin E was well tolerated, with no significant adverse events as compared with placebo. This finding provides some reassurance for the conduct of large, longer-term trials to address unanswered questions regarding vitamin E, such as its possible effects in preventing cancer.

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REFERENCES

1. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320:915-24.
2. Witztum JL. The oxidation hypothesis of atherosclerosis. Lancet 1994; 344:793-5.
3. Carew TE, Schwenke DC, Steinberg D. Antiatherogenic effect of probucol unrelated to its hypercholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci U S A 1987;84:7725-9.
4. Verlangieri AJ, Bush MJ. Effects of δ-α-tocopherol supplementation on experimentally induced primate atherosclerosis. J Am Coll Nutr 1992;11: 131-8.
5. Williams RJ, Motteram JM, Sharp CH, Gallagher PJ. Dietary vitamin E and the attenuation of early lesion development in modified Watanabe rabbits. Atherosclerosis 1992;94:153-9.
6. Knekt P, Reunanen A, Jarvinen R, Seppanen R, Heliovaara M, Aromaa A. Antioxidant vitamin intake and coronary mortality in a longitudinal population study. Am J Epidemiol 1994;139:1180-9.
7. Gaziano JM, Manson JE, Branch LG, Colditz GA, Willett WC, Buring JE. A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly. Ann Epidemiol 1995;5:255-60.
8. Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y, Bostick RM. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. N Engl J Med 1996;334:1156-62.
9. Klipstein-Grobusch K, Geleijnse JM, den Breeijen JH, et al. Dietary antioxidants and risk of myocardial infarction in the elderly: the Rotterdam Study. Am J Clin Nutr 1999;69:261-6.
10. Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC. Vitamin E consumption and the risk of coronary disease in women. N Engl J Med 1993;328:1444-9.
11. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. N Engl J Med 1993;328:1450-6.
12. Hodis HN, Mack WJ, LaBree L, et al. Serial coronary angiographic evidence that antioxidant vitamin intake reduces progression of coronary artery atherosclerosis. JAMA 1995;273:1849-54.
13. Blot WJ, Li JY, Taylor PR, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. J Natl Cancer Inst 1993;85:1483-92.
14. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. N Engl J Med 1994;330:1029-35.
15. Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study. Lancet 1996;347: 781-6.
16. GISSI-Prevenzione Investigators (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Lancet 1999;354:447-55.
17. The Heart Outcomes Prevention Evaluation Study Investigators. Effects of an angiotensin-converting–enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. N Engl J Med 2000;342:145-53.
18. The HOPE Study Investigators. The HOPE (Heart Outcomes Prevention Evaluation) Study: the design of a large, simple randomized trial of an angiotensin-converting enzyme inhibitor (ramipril) and vitamin E in patients at high risk of cardiovascular events. Can J Cardiol 1996;12:127-37.
19. Rapola JM, Virtamo J, Ripatti S, et al. Randomised trial of α-tocopherol and β-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. Lancet 1997;349:1715-20.
20. Virtamo J, Rapola JM, Ripatti S, et al. Effect of vitamin E and beta carotene on the incidence of primary nonfatal myocardial infarction and fatal coronary heart disease. Arch Intern Med 1998;158:668-75.
21. Yusuf S, Peto R, Lewis J, Collins R, Sleight P. Beta blockade during and after myocardial infarction: an overview of the randomized trials. Prog Cardiovasc Dis 1985;27:335-71.

22. Steinberg D. Clinical trials of antioxidants in atherosclerosis: are we doing the right thing? Lancet 1995;346:36-8.
23. Hennekens CH, Buring JE, Manson JE, et al. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. N Engl J Med 1996;334:1145-9.
24. Lonn EM, Yusuf S, Doris CI, et al. Study design and baseline characteristics of the Study to Evaluate Carotid Ultrasound Changes in Patients Treated with Ramipril and Vitamin E: SECURE. Am J Cardiol 1996;78:914-9.
25. Upston JM, Terentis AC, Stocker R. Tocopherol-mediated peroxidation of lipoproteins: implications for vitamin E as a potential antiatherogenic supplement. FASEB J 1999;13:977-94.
26. Hercberg S, Galan P, Preziosi P, et al. Background and rationale behind the SU.VI.MAX Study, a prevention trial using nutritional doses of a combination of antioxidant and minerals to reduce cardiovascular diseases and cancers: SUpplementation en VItamines et Mineraux AntioXydants Study. Int J Vitam Nutr Res 1998;68:3-20.
27. MRC/BHF Heart Protection Study of cholesterol-lowering therapy and of antioxidant vitamin supplementation in a wide range of patients at increased risk of coronary heart disease death: early safety and efficacy experience. Eur Heart J 1999;20:725-41.
28. Jha P, Flather M, Lonn E, Farkouh M, Yusuf S. The antioxidant vitamins and cardiovascular disease: a critical review of epidemiologic and clinical trial data. Ann Intern Med 1995;123:860-72.

ANNEX C

:linical Trials Report [abstract]

obert A. Hegele, MD

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ngiotensin-converting Enzyme (ACE) Inhibition in the Secondary Prevention of Vascular Disease: The leart Outcomes Prevention Evaluation (HOPE) Trial and Its Substudies

OPE Study Investigators : **Effects of an angiotensin-converting enzyme inhibitor, ramipril, on cardiovascular vents in high-risk patient.** *N Engl J Med* 2000, **342:** 145-153.

OPE Study Investigators : **Vitamin E supplementation and cardiovascular events in high-risk patients.** *N Engl J Mec* 000, **342:** 154-160.

OPE Study investigators : **Effects of ramipril on cardiovascular and microvascular outcomes in people with iabetes mellitus.** *Lancet* 2000, **355:** 253-259.

ıtroduction: The landmark Heart Outcomes Prevention Evaluation (HOPE) study was initiated in the early 1990s in large part to clarify the th ontroversial issue regarding the benefit of vitamin E in the reduction of cardiovascular events in high-risk patients with vascular disease. For a ariety of reasons, a two-by-two factorial study design was settled upon, with the intention of evaluating both vitamin E and the ACE inhibitor ımipril in these patients. A priori substudies, including one to evaluate the development of cardiovascular events and overt nephropathy in ubjects with diabetes were also part of the study design.

lethods: To evaluate the effects of both vitamin E (400 IU/d) and ramipril (10 mg/d), 9297 patients from multiple centers were randomized in a X 2 study design. Subjects were 55 years of age and older, with cardiovascular disease or diabetes plus one other risk factor, and did not ha low ejection fraction or heart failure. Subjects were randomized to receive either ramipril alone, vitamin E alone, ramipril plus vitamin E, or lacebo. The primary outcome was a composite of myocardial infarction (MI), stroke, or death from cardiovascular causes. In addition, the evelopment of overt nephropathy was a main outcome in the substudy of subjects with diabetes.

esults: The study was stopped 6 months early (after 4.5 years) by the independent data safety and monitoring board because of both a lack ffect of vitamin E and a consistent benefit of ramipril compared with placebo. Treatment with ramipril was associated with significant reduction i the relative risk of primary composite endpoints (0.78, $P<0.001$), and other individual primary endpoints such as cardiovascular death (0.74, <0.001), MI (0.80, $P<0.001$), stroke (0.68, $P<0.001$), in addition to revascularization procedures (0.85, $P=0.002$) and all-cause mortality (0.84, =0.005). In contrast, there was no significant difference in any endpoint between the vitamin E and placebo groups (all 95% CI, spanned 1.0). ıterestingly, there was a suggestion of a possible benefit in the secondary outcome of development of cancer, so the study is being extended valuate the effect of vitamin E on cancer incidence. The results of the diabetic substudy for the ramipril-treated subjects were consistent with ıose of the overall study, showing relative risk of approximately 0.8 for all major endpoints. Importantly, the relative risk of overt nephropathy as reduced in the ramipril-treated group (0.76, $P<0.03$). All studies showed no significant increase in most side effects or adverse events in th eated groups.

iscussion: The results of these studies suggest that ramipril 10 mg/day significantly reduces rates of death, MI, stroke, and other important ardiovascular endpoints in a broad range of high-risk patients, including diabetic subjects, who are not known to have a low ejection fraction o eart failure. Conversely, treatment with vitamin E 400 IU/day had no apparent influence of cardiovascular outcomes.

ditor's Comments

is of interest that most of the attention given to the HOPE trial while it was ongoing, both from presentations by the investigators and from the xpectations of the patients, was focussed on the vitamin E component. This was not surprising, given the substantial preclinical scientific nderpinnings and the attractiveness of a "natural" antioxidant strategy to influence cardiovascular outcomes. The negative result of the vitamir arm in HOPE was thus disappointing, and questions still linger about the appropriateness of the dose and duration of treatment with vitamin I nd whether concomitant administration of vitamin C or other antioxidants would have made any difference. However, due to prudent xperimental design, the study was redeemed, in a sense, by the ramipril arm, and these practice-changing findings will have important and ısting clinical implications.

he strength of a clinical trial with a simple design is that significant results have a high likelihood of clinical applicability and utility, and thus a ıore immediate benefit for healthcare delivery and population health. However, this same simplicity of design also represents a major ıortcoming for theoretically-minded individuals who wish to understand the results in more basic or mechanistic terms. This type of nderstanding is virtually impossible to obtain from a trial such as HOPE. For example, there has been some argument as to how much of the enefit of ramipril in HOPE was the result of blood pressure lowering or some other effect of ACE inhibitors, or whether the results represent a ass effect of these medications. The HOPE study cannot hope to resolve this. For a clinician, such issues might be irrelevant–the patient sitti i the office today needs evidence-based treatment immediately. However, a researcher who is concerned with defining new mechanisms and isease pathways can never be satisfied by the lack of intermediate molecular cellular and biochemical information in these studies. It is the efinition of such mechanisms that will provide targets for novel intervention strategies that will have additional and profound effects on disease ɛduction in the future.

he HOPE investigators indicated that their study had its beginnings within the last decade, inspired by suggestive clinical observations that ngiotensin-converting enzyme (ACE) inhibition would be beneficial in cardiovascular risk reduction. This might have seemed true to them with ıe context of the clinical-trials literature. However, ACE inhibitors did not arise from a vacuum. The HOPE trial was merely the last step of a rocess that began more than 30 years ago. At that time, the characterization of the basic science of ACE suggested that it was a good harmacologic target. Without the basic understanding of the enzymology and physiology of ACE, there would be no pharmacologic ACE

ANNEX D

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Review article

The use of antioxidant supplements in coronary heart disease

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Abstract

There is clear evidence of lipoprotein oxidation in atherosclerotic lesions. Animal studies and observational prospective human cohort studies have been interpreted as supporting a role for antioxidants in the prevention of coronary heart disease (CHD). However, firm recommendations to take antioxidant supplements to treat or prevent CHD require evidence derived from randomised controlled studies. In primary prevention studies, low dose α-tocopherol does not reduce the incidence of coronary events (ATBC study), and β-carotene either has no effect or increases the incidence of coronary events and cancer death (ATBC, CARET, Physician's Health studies). Secondary preventions, those with smaller populations and shorter duration of follow up have shown some benefit from α-tocopherol (CHAOS, SPACE), but larger randomised studies indicate no benefit from treatment with α-tocopherol (HOPE, GISSI, PPP). Recent studies with antioxidant combinations also show no benefit (HATS, MPS). On the basis of these data, supplements of α-tocopherol and β-carotene cannot be recommended for the treatment or prevention of CHD. Fundamental and applied research may yet find a role for antioxidant supplements in the treatment of coronary disease. However, this will require positive results from combined antioxidant studies currently in progress, and the targeting of oxidative processes that operate in the artery wall and cause or contribute to disease.

Keywords: Lipoprotein oxidation; Atherosclerotic lesions; β-carotene; Antioxidants; α-tocopherol; Vitamin E; coronary; atherosclerosis

1. Introduction

The role of lipoprotein oxidation, and particularly oxidation of low-density lipoprotein (LDL), in atherogenesis has been the subject of intense investigation. Major reviews of LDL oxidation have been published, each concentrating upon different aspects of atherosclerosis research (e.g. [1–6]. The aim of this article is to review randomised controlled studies investigating the clinical use of antioxidant supplements to prevent or treat coronary heart disease (CHD). In the first section, some of the background data suggesting clinical benefit of antioxidant supplementation is presented. These include selected epidemiological nutritional studies, prospective cohort studies, intervention studies measuring surrogate endpoints such as carotid-intima media thickness (IMT), and laboratory studies. Recent important randomised controlled trials sufficiently powered to measure altered cardiovascular outcomes are the main focus of this article and provide the basis for current recommendations. Finally, we attempt to reconcile apparently contradictory data in order to suggest future directions for this important clinical question.

2. Nutritional aspects of the attribution of cardiovascular protection to individual antioxidants

Absolute rates of CHD between countries with similar mean serum cholesterol varies, and may relate to factors such as consumption of saturated fat and antioxidants, and non-dietary factors such as cigarette smoking [7]. Many investigators have found that populations with low rates of CHD consume diets rich in antioxidants such as vitamin E [8] and this agrees with studies

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measuring plasma levels of some antioxidants [9]. Gey found that the apparent clinical benefit was great despite only small difference in apparent plasma concentrations of α-tocopherol in high- and low-intake population [8]. Some studies have also identified that flavonoids contained in apples, onions and tea, appear to confer protection against vascular disease [10] although overviews of multiple studies provide conflicting results [11].

Plasma antioxidant concentrations may serve as an indirect measure of antioxidant intake as well as a marker of consumption of healthy whole foods. Confounding non-dietary lifestyle variables may also contribute to apparent favourable effects in non-randomised studies. Finally, there are many different antioxidants within single food groups, and different antioxidants within a so-called single class of antioxidants. For example, apparently simple food stuffs such as apples and tea contain various diverse flavonoids and non-flavonoid micronutrients, and their relative abundance will vary between food stuffs and between regions, and all may be bioactive in ways other than acting as antioxidants. To attribute the atheroprotective effect of these foods to the antioxidant capacity of a single identified flavonoid would be similar to the attribution of the atheroprotective effects of the Mediterranean diet to vitamin E consumption alone. The Lyon Heart Study [12], found a clear reduction in recurrence of CHD after randomising patients to an entire diet (containing increased fibre intake, omega 3 polyunsaturated fatty acid intake, as well as fruit, and vegetable consumption). Although such dietary change would provide antioxidants, diverse bioactive compounds and nutrients may contribute to favourable effects of such a diet.

2.1. Prospective cohort studies

Several case control studies, for example [9], have found an inverse association for plasma vitamin E, vitamin C, β-carotene and the risk of angina. Nested case control studies measuring baseline plasma levels of antioxidants many years after sample collection have found variable results and are difficult to interpret as they are potentially complicated by sample degradation following prolonged storage.

Prospective cohort studies have correlated high self-reported intakes of antioxidants with low rates of CHD. For example, the 87 000 population Nurse's Health Study [13] showed benefit with supplemental vitamin E 100 IU per day but not with low dose supplements or with high natural dietary intake of vitamin E. The 40 000 population male health professional study also found that 100 IU per day supplemental vitamin E reduced rates of CHD, that 60 IU per day conferred significant benefit relative to 7.5 IU day, and that β-carotene was protective in current and past smokers [14]. A slightly contrary result was derived from a population of 35 000 women [15]. In this study, an apparently modest variation in dietary intake of vitamin E from <4.9 IU per day (lowest quartile) to 9.6 IU per day (highest quartile) was associated with a significant reduction in cardiovascular risk, and there was no benefit attributable to vitamin E (or vitamin A and C) supplements [15].

Although at first glance prospective cohort data are supportive of a role for vitamin E in protecting against CHD, significant discrepancies exist in these data. Most apparent is that the benefit attributable to the small amount of vitamin E ingested within food is far greater than the dose required for benefit from vitamin E taken as a supplement. Differences between studies in terms of dose and source of vitamin E (food vs. supplement) are unexplained but are potentially important. They may indicate that antioxidants act as surrogate measures of healthy diets, that their benefit only occurs in the presence of other unidentified micronutrients, or that baseline patient antioxidant consumption and/or oxidative stress may vary and may determine the effect of supplements [16].

3. Measures of lipid peroxidation

3.1. In vitro LDL oxidation

There is unequivocal evidence of lipid and protein oxidation in atherosclerotic lesions (see reviews cited above). In vitro studies have indicated that a number of antioxidants, including water-soluble vitamin C, and lipid-soluble vitamin E (mostly α-tocopherol and lesser amounts of γ-tocopherol), ubiquinol-10, lycopene and carotenoids can inhibit the oxidation of LDL. However, depending on the precise measurements and experimental systems used, even vitamin E, which is conventionally considered to be an anti-oxidant, can demonstrate a pro-oxidant activity [4]. Within plasma, water-soluble antioxidants and lipid-soluble antioxidants can co-operate in preventing the oxidation of emulsions such as lipoproteins, and within emulsions ubiquinol-10 and α-tocopherol can co-operate to prevent oxidation. As in vitro LDL oxidation studies remove LDL from its natural environment, the biological relevance of in vitro LDL oxidation is uncertain. The natural environment in which LDL oxidation may be expected to take place, the arterial wall, is extremely difficult to replicate as it remains biochemically poorly defined, and atherosclerotic plaque itself contains large quantities of antioxidants including α-tocopherol and vitamin C [17]. Moreover there are diverse oxidative processes identified within atherosclerotic plaque [3], and some of these will likely be differentially responsive to individual antioxidants.

Another important issue affecting the relationship between in vitro LDL oxidation and human nutrition studies is that 'antioxidants' are not simply antioxidants. For example, vitamin E has antithrombotic effects [18], phenolic compounds present in red wine inhibit platelet aggregation [19], and flavonoids are anti-inflammatory [20]. Thus some antiatherogenic effects of bioactive molecules may be unrelated to their modulation of LDL oxidation, even if this process were to be relevant to atherogenesis [21,22].

3.2. F_2-isoprostanes and the in vivo effects of antioxidants

F_2-isoprostanes are prostaglandin-like compounds produced during the free radical-catalysed oxidation of arachidonic acid which have the important potential to serve as non-invasive and direct markers of in vivo oxidative stress [23]. Urinary levels of F_2-isoprostanes are elevated in patients with hypercholesterolaemia [24], diabetes [25], and smokers [26]. Falls in urinary F_2-isoprostanes were observed in cigarette smokers taking vitamin C or a combination of vitamin C and E but not vitamin E alone [27], in hypercholesterolaemic subjects supplemented with vitamin E [24], and in healthy adults [28]. Supplemental vitamin E prolonged mean LDL oxidation lag time but, paradoxically, further increased F_2-isoprostanes in cigarette smokers consuming a high polyunsaturated fat diet [29]. Flavonoid consumption in onions and tea had no significant effect on plasma F_2-isoprostane concentrations and MDA–LDL autoantibody titer [30].

However, most of these studies described to date are uncontrolled and conflicting positive and negative results between studies may point to variations in assays of isoprostanes or population differences. Whether the negative results with vitamin E relate to the lack of efficacy of the supplement (as suggested by large randomised studies described below) or whether they relate to the complexity of factors affecting isoprostane production and/or metabolism is unclear. It remains similarly unclear to what extent plasma or urinary isoprostanes reflect LDL oxidation. Nevertheless, the general principle of assessing in vivo oxidative stress and in vivo response to antioxidants is an important one requiring further development.

4. Surrogate endpoint studies—intima-to-media thickness

Carotid intima-to-media thickness (IMT) measured by ultrasound is commonly used as a surrogate marker of atherosclerotic disease, and traditional risk factors for ischaemic heart disease and antioxidant intake relate to IMT (e.g. [31]). Sometimes referred to as a measure of 'preintrusive' atherosclerotic disease, it correlates weakly with the prevalence and extent of coronary artery disease measured by arteriography [32], but appears useful for longitudinal evaluation of study populations. In a self-selected population within the CLAS study, patients taking high dose vitamin E and not on lipid-lowering medication had less IMT progression than those taking low dose vitamin E [33]. Randomised studies using IMT and various drugs have recently been described. For example, pravastatin was shown to inhibit progression of IMT [34], whereas ramipril (5–10 mg) did not [35]. In the SECURE substudy of the HOPE study (see below), 732 patients with vascular disease or diabetes and at least one other risk factor for coronary disease were investigated for IMT progression. This was dose-dependently reduced by ramipril, but unaffected by vitamin E [36]. The ASAP study randomised 520 men and women to vitamin E (91 mg per day), 250 mg per day slow release vitamin C, or both for 3 years. The rate of progression of IMT was reduced only amongst men taking both vitamin E and vitamin C, was unaffected by the consumption of any antioxidant(s) in women, and was unaffected by the taking of either antioxidant alone in men [37].

Data pertaining to antioxidants and IMT appear to parallel the observational studies of vitamin E intake, in which benefits appear most marked in self-selected populations. Of only two randomised studies to date, SECURE and ASAP suggest a null effect of vitamin E. The apparent effect of vitamins C and E in combination in the ASAP study appears less robust as it occurred in men only, but suggests that studies with antioxidant combinations are worthy of further clinical investigation.

5. Randomised controlled studies

5.1. General issues and limitations

Variations in plasma concentrations of vitamin E identified in nutritional studies as relevant to the incidence of CHD incidence are very small (i.e., in the low μmol/l range), and are exceeded by most studies using antioxidant supplements. However, some prospective cohort studies found that a dose of vitamin E of ≥ 100 IU per day was required to reduce rates of CAD [14]. As a result, the 50 mg apo E per day used in the primary prevention ATBC study (see below and Table 1) were subsequently considered to be too low. Although this view is inconsistent with the higher increase in plasma vitamin E observed in the primary prevention compared with the nutritional studies, the possibility that higher doses matching those used in prospective cohort studies may have provided protection cannot be excluded. Secondly, whereas the follow up over 5 years used in the primary prevention studies is

Table 1
Summary of major randomised studies

Study title (reference)	Design/follow-up	Population[a]	Antioxidant/active treatment	Major outcomes
Linxian [38]	Open label 5.3 year no true placebo	$N = 29\,584$, M and F, 40–69 year	1, Retinol + zinc; 2, Riboflavin + niacin; 3, Vitamin C (120 mg) + molybdenum, β-C (15 mg) + E (30 mg) + selenium	↓ overall death (primarily cancer-related): (RR0.91, 95% CI 0.84–0.99) from treatment group 4; no effect cerebrovascular death; CHD not reported
ATBC [39], ATBC subgroups [41]	DB, PC, 2 × 2, 6.1 year	$n = 29\,134$, M smokers; 50-69 year, primary prevention $n = 27\,272$ secondary preventionn = 1862	1, E (50 mg = 55 IU), 2, β-C (20 mg), 3, E + β-C	β-C trend ↑ lung cancer, (RR 1.02, 95% CI 0.95–1.09), β-C ↑ overall mortality, (RR 1.08, 95% CI 1.01–1.06), β-C, E no effect on overall primary prevention AMI, or overall secondary prevention AMI
CARET[42]	DB, PC, 4 year	$N = 18\,314$, M and F past or present smokers, asbestos exposure (primary prevention)	β-C (30 mg) + vit A (25000 IU retinol)	β-C + vit A ↑ lung cancer, (RR 1.28, 95% CI 1.04–1.57, $P = 0.02$), β-C + vit A ↑ all cause mortality, (RR 1.17, 95% CI, 1.03–1.33, $P = 0.02$), β-C + vit A trend to ↑ CV death, (RR 1.26, 95% CI 0.99–1.61)
Physician health [43]	DB, PC, 12 year for β-c, 2 × 2	$N = 22\,071$, (primary prevention)	1, β-C (50 mg alt days). 2, Aspirin	β-C no effect CV death, AMI, or all cause mortality CV death-(RR 1.20, 0.93–1.11), aspirin arm stopped early-↓ AMI
CHAOS [45]	DB, PC, 1.4 year (range 3 day–3 year)	$N = 2002$, with coronary disease (secondary prevention)	E 800 or 400 IU	E ↓↓ AMI non-fatal (RR 0.23, 95% CI 0.11-0.47, $P < 0.001$) E Trend ↑ cardiovascular death (RR 1.18, 95% CI 0.62–2.27, $P = 0.6$)
GISSI-P [46].	OL, PC, 3.5 year, 2 × 2	$N = 11\,234$, AMI within 3 months (secondary prevention)	1, E (300 mg = 330 IU). 2, Fish oil (1.0 g)	E no effect AMI + death + stroke (0.88 (95% CI 0.75–1.04), Fish oil ↓ AMI + death + stroke, 0.80 (95% CI 0.68–0.95)
HOPE [48,49].	DB, PC, 4.5 year, 2 × 2	$N = 9541$, High risk (primary, secondary prevention)	1, E (400 IU). 2, Ramipril	E no effect AMI + CV death + stroke (RR 1.05, 95% CI 0.95–1.16, $P = 0.33$). Ramipril ↓ AMI + CV death + stroke (RR 0.78, 95% CI 0.70–0.86 $P < 0.001$)
SPACE [50]	DB, PC, 2 year	$N = 196$. Haemodialysis patients (secondary prevention)	E (800 IU)	E ↓↓ combined endpoint of AMI + stroke + PVD + unstable angina (RR 0.46, 0.27–0.78, $P = 0.014$)
PPP [44]	OL, PC, 3.6 year, 2 × 2	$N = 4495$. High risk for CV disease (primary prevention)	1, E (300 mg); 2, Aspirin (100 mg)	E no effect (RR 1.07, 95% CI 0.74–1.56). Aspirin ↓ AMI + CV death + stroke (RR 0.77, 95% CI 0.62–0.95)
HATS [53]	DB, PC, 3.5 year, 2 × 2	$N = 160$. With coronary disease (secondary prevention)	1, Simvastatin-niacin (S-N). 2, Antioxidants (E 800 IU, C 1000 mg, β-C 25 mg, selenium 100 μg)	S-N ↑ HDL2, ↓ angiographic progression (from 3.9 to 0.7%, $P = 0.004$) ↓ events (from 24 to 3%, $P = 0.02$) antioxidants-↓ HDL2, ↓ angiographic progression (3.9 to 1.8% $P = 0.16$, NS), inhibit effect of S-N on events, progression, HDL
HPS [55]	DB, PC, 5.5 year, 2 × 2	$N = 20\,356$. High risk (primary, secondary prevention)	1, Simvastatin (40 mg). 2, Antioxidants (E 600 mg, C 250 mg, β-C 20 mg)	Simvastatin ↓ AMI + CV death + stroke (RR 0.72, $P = 0.0001$). Antioxidants no effect (RR 1.0)

[a]. Primary and secondary prevention defined on basis of history of myocardial infarction, except for CHAOS where based on coronary angiography. Abbreviations: DB, double-blind; PC, placebo-controlled; E, vitamin E; C, vitamin C; β-c, β-carotene; AMI, acute myocardial infarction; OL, open label, CV, cardiovascular; 2 × 2, 2 × 2 factorial design comparing placebo, agent A, agent B, and combination of agent A and agent B; PVD, peripheral vascular disease; simvastatin-niacin S-N.

conventional for pharmaceutical studies, the results obtained do not preclude benefits of antioxidants which may derive from a lifetime of dietary intake. Thirdly, and perhaps most importantly, study entry criteria have generally not included in vivo quantitative indices of oxidative stress or anti-oxidant deficiency, so that individual response to antioxidants is unpredictable and not measured [16].

With these caveats, the best of these studies are of high quality (on the basis of consistency of randomisation, consistency of effect, and patient numbers) and are sufficiently powered to detect altered cardiovascular outcomes.

5.2. Major clinical studies (summarised in Table 1)

5.2.1. Primary prevention

Four combinations of nutrients: (A) retinol and zinc; (B) riboflavin and niacin; (C) vitamin C and molybdenum; and (D) β-carotene (15 mg), vitamin E (30 IU),

and selenium (50 μg) were investigated in the randomised, open label Ling Xian study for effects on cancer mortality in 29 000 adult Chinese over a 5 year period [38]. This study found a small but significant decrease in total mortality attributable to reduced incidence in cancer, especially of the stomach, in those receiving the combination (D). There was no significant effect of any combination on cardiovascular death, and there was no true placebo group, limiting the relevance of the data.

In the Finnish ATBC (α-tocopherol, β-carotene) study [39–41], there was no effect of antioxidants on incidence of fatal or nonfatal myocardial infarction. There was a higher incidence of lung cancer among the men who received β-carotene than among those who did not. An increased risk of haemorrhagic stroke and decreased risk of prostate cancer were identified with vitamin E but are of uncertain importance, given their absence in the GISSI, CHAOS, or HOPE studies. In the β Carotene and Retinol study (CARET), β-carotene and retinyl palmitate significantly increased the risk of lung cancer and there was a non-statistically significant trend toward increased cardiovascular death [42]. In the Physician's health study, there were no differences in the incidence of cancer, cardiovascular disease, myocardial infarction, stroke or overall mortality attributable to carotene [43].

These studies collectively negate the apparent benefit attributed to carotene in prospective cohort studies. Indeed ATBC and CARET suggested a harmful effect of carotene. The Physician's Health study suggests retinoic acid may have contributed to adverse events in BCARET, and confirms at best a null effect of carotene on cardiovascular outcomes.

In the collaborative primary prevention project (PPP), open label low dose aspirin and vitamin E were investigated in general practice [44]. Stopped prematurely, because of the evidence of favourable effects of aspirin from other studies such as the Physician's health study, it confirmed aspirin prevented cardiovascular events, and showed vitamin E had no effect.

5.2.2. Secondary prevention

In the Cambridge Antioxidant Study (CHAOS) study [45] vitamin E reduced the risk of non-fatal AMI, but caused a non-significant increase in fatal AMI. This study has a number of limitations. Some baseline characteristics were not balanced, raising questions as to the randomisation process; the extent of reduction was exceptional given the small number of patients and short and variable follow up; and the reduction in non-fatal AMI was at odds with the trend of effect on fatal AMI.

In the large GISSI-P study, n-3 polyunsaturated fatty acids (PUFA, 1 g per day, 2:1 docosahexaenoic acid: eicosapentanoic acid) reduced the relative risk of the combined endpoint of cardiovascular death, non-fatal myocardial infarction, and stroke to 0.80 (95% interval 0.68–0.95), whereas vitamin E showed a non-significant trend (RR 0.88, 95% CI 0.75–1.04) [46]. The interpretation of these results is complicated however, because, the GISSI-P study is confounded by substantial discontinuation rates and dietary changes (with conversion to higher fish, vegetable and olive oil intake), the lack of independent monitors, and the open label treatment. Although the effect of vitamin E might have reached statistical significance had the rate of discontinuation and incidence of dietary change not been so high, this is speculative. There was no evidence of interaction by combining vitamin E and PUFA, which might be expected if PUFA were protected from oxidation by vitamin E, as would be expected from some in vitro LDL oxidation studies. The fact that n-3 PUFA offered protection is not immediately consistent with the oxidation theory of atherosclerosis, as such supplements may be expected to increase rather than decrease the susceptibility of LDL to oxidation.

The difference in outcome between GISSI and CHAOS may relate to the basal nutritional status of the population studied. A Northern European/British diet may be low in natural antioxidants, and regional over-expression of a polymorphism for the nitric oxide synthase gene [47] may have augmented the responsiveness of the CHAOS population to vitamin E. If local factors such as diet and genotype, as well as background prescription of ACE-inhibitors, aspirin, and lipid-lowering agents determine the benefit of antioxidants, extrapolation of these results to the general population may be precluded.

In the Heart Outcomes Prevention Evaluation Study (HOPE), patients with known cardiovascular disease, or diabetes plus another risk factor were treated with ramipril or vitamin E or both or placebo. Those treated with ramipril had a substantially reduced risk of the combined primary endpoint of AMI, stroke or cardiovascular death, but treatment with vitamin E exerted no benefit [48,49]. As this study used 400 IU vitamin E per day, which matches the dose administered in the CHAOS study, and combined a larger cohort of patients with longer (4 years) follow up, the negative result is compelling. The CHAOS and HOPE studies differed in baseline medication usage. Compared with CHAOS, more patients in the HOPE study were taking aspirin (76 vs. 54.4%) fewer were taking calcium antagonists (46.3 vs. 69.2%), 29% were taking lipid-lowering agents and half were taking the ACE inhibitor ramipril (usage of ACE inhibitor and lipid-lowering agents unspecified in CHAOS). Finally, CHAOS was restricted to the UK whereas HOPE was a multinational study. Some of these therapeutic and population differences may have contributed to the negative result in HOPE. However, as aspirin, lipid-lowering agents and ACE-inhibitors are

commonly prescribed for subjects with known coronary disease, any proven benefit of antioxidant supplements must be shown to be incremental to the use of such established therapeutic agents.

In a very small population of haemodialysis patients (SPACE study) vitamin E achieved a major decrease in rates of AMI (RR 0.3, (0.11–0.78), $P = 0.016$) [50]. The extent of such decrease is remarkable and unexpected given the results of previous studies and the very short duration of follow up. This result may be attributable to small patient numbers or special characteristics of the renal failure population. For example, there is some evidence for a deficiency in plasma antioxidants in haemodialysis patients [51,52], though this requires substantiation.

The 3-year, double-blinded, placebo-controlled, secondary prevention HDL-Atherosclerosis Treatment study (HATS) has recently been published [53]. 160 subjects with known coronary artery disease, low HDL, 'normal' LDL, were randomised to simvastatin plus niacin, antioxidant supplements (combination of 800 IU vitamin E, 1000 mg vitamin C, 25 mg β-carotene, and 100 μg selenium per day), both simvastain-niacin and antioxidants, or neither. The primary end-points were arteriographic change in coronary stenosis and occurrence of a first cardiovascular event. Antioxidant supplementation increased plasma concentrations of vitamins E, C, and β-carotene, and increased the resistance of plasma LDL to in vitro oxidation. Simvastatin-niacin lowered LDL by 42%, elevated total HDL by 26 and HDL2 by 60.5%, reduced the average rate of progression of stenosis from 3.9 (with placebo) to 0.4% ($P < 0.001$), and reduced the rate of clinical events from 24% (with placebo) to 3% ($P = 0.03$). In contrast, antioxidants non-significantly reduced the incidence of clinical endpoints relative to placebo ($P = 0.16$), decreased plasma HDL2 by 15.4%, and non-significantly reduced the average rate of progression of coronary stenosis to 1.8%. Of particular concern, antioxidants inhibited the favourable effects of simvastatin-niacin on HDL2 ($P = 0.02$), angiographic lesion progression ($P = 0.02$) and clinical events ($P = 0.13$, NS). This study suggests that HDL lowering may mediate unfavourable effects of antioxidants and this parallels earlier data with probucol [54].

The MRC/BHF Heart Protection Study (HPS), addressing the effect of antioxidant combinations in addition to lipid lowering therapy in patients with known coronary disease, has been presented recently in preliminary form [55]. Over 20 000 subjects were randomised to 40 mg simvastatin, antioxidant combination (600 mg vitamin E, 250 mg vitamin C, 20 mg β-carotene), both or neither for a mean of 5.5 years. Whereas simvastatin reduced all cause mortality by 12% ($P < 0.001$) and cardiovascular events by 24% ($P < 0.0001$), antioxidants had no effect whether alone or when combined with simvastatin.

5.3. Summary of randomised controlled studies

The strength of the association between food antioxidant consumption and the prevention of coronary events is strongest in observational studies, which are unfortunately confounded by self-selection of patients and co-consumption of other nutrients in whole foods. In IMT studies, vitamin E alone exerted no effect in most randomised controlled studies, but an isolated observation suggests that administration of both vitamin E and vitamin C may be beneficial. In large, well-designed randomised placebo-controlled studies powered to detect clinical events (ATBC, CARET, Physician's Health, HOPE, GISSI, PPP, HPS) the data overall indicate a null effect of vitamin E, and a null or adverse effect of β-carotene. Two secondary prevention studies with relatively small numbers and short follow up, CHAOS and SPACE suggest that certain sub-populations may benefit from vitamin E supplements, but criteria for the identification of these subgroups require clear definition and validation. Although recent AHA guidelines [56] preceded the publication of some of these studies, the recommendation that diets rich in antioxidants, but not antioxidant supplements, be advocated, has only been strengthened with the publication of HOPE, PPP, and HPS. HOPE, GISSI, PPP, HPS and HATS also demonstrate that any apparent benefit of antioxidant consumption must be related to treatments with proven efficacy, such as statins, ACE inhibitors and aspirin, which many of the high-risk population will be taking.

6. Revisiting lipoprotein oxidation and atherogenesis

6.1. Reconciling lipoprotein oxidation in the arterial wall, nutritional studies and randomised controlled studies of antioxidant supplements

It is possible to reconcile the large body of pre-clinical data, and nutritional observations with recent negative randomised studies. There is no doubt that lipoprotein oxidation is evident in the arterial wall, and that oxidised lipids and proteins exert many potential biological effects. What is less clear is whether these effects are causal for (rather than consequential to) atherosclerosis or for any of its complications. Animal studies currently dissociating anti-atherogenic and antioxidant effects of pharmacological agents may help resolve these issues [22,57]. Selected targeting of those processes for which oxidation is causal may permit benefits of antioxidants to be revealed. It is also not clear which types of oxidants are responsible for the

oxidative modification of important targets. Importantly, vitamin E is an effective scavenger of one-electron oxidants (i.e. radicals), yet it does not protect LDL from radical oxidants under all conditions [58]. In addition, vitamin E offers little protection against two-electron oxidants, that are increasingly implemented during the early stages of atherosclerosis [59,60]. Therefore, a re-evaluation of the relevant biological consequences of oxidation, identification of the oxidative processes operative in the arterial wall at different stages of atherogenesis, as well as evaluation of the consequences of their inhibition in animal models is required.

Nutritional data indicating an important role for diet and environment in atherosclerosis risk are also compelling. Most likely, differences between randomised studies and nutritional observations derive from assuming the benefits of whole foods can be attributed to individual micronutrients and the confounding of observational data. The results with β-carotene are highly relevant here, given the unequivocally non-beneficial effect of this supplement, but the generally favourable effects attributable to whole diets that contain β-carotene. Results from the Lyon study and the GISSI studies support the potential cardiovascular benefit of dietary change.

6.2. Future directions

Several major issues must be addressed in future studies before antioxidant supplements can be recommended. First, criteria for the identification of patient subgroups requiring or deficient in antioxidants must be established. It is impossible to evaluate the relevance of data such as that in the Space study [50] to the population as a whole without careful characterisation of the patient population, and identifying if the study population is in some way unique. Similar considerations apply to the potential interaction of vitamin E with the NOS genotype in the UK CHAOS study [47]. Where possible, the effects of antioxidant supplementation should be measured in plasma, and monitored by in vivo response to antioxidant therapy, such as by measuring plasma isoprostanes in specialised laboratories. Second, the potential for multiple antioxidants to interact needs to be addressed.

The recently released HPS and HATS studies, and the currently in progress Women's Antioxidant and Cardiovascular Study will resolve the potential for multiple antioxidants to protect against atherosclerosis. This may approximate the situation in whole foods where multiple micronutrients co-exist, and may allow co-operative antioxidant activity, as can be observed in vitro, to take effect. However, given the evidence to date from HPS [55] and HATS [53], it is unlikely that antioxidant combinations will substantially alter the current conclusion of null effect derived from studies of single antioxidants.

6.3. Conclusions

Although absence of harm with recent large vitamin E studies is reassuring, recommendations to take antioxidant supplements require convincing proof of positive effect. Diets rich in fruits, vegetables, whole grain cereals, dietary fibre and polyunsaturated fats, and low in added salt, saturated and *trans*-fatty acids (which may be replaced by polyunsaturated and monounsaturated fats) are currently recommended. However, current controlled data do not provide grounds for recommending antioxidant supplements for the prevention or treatment of CHD.

References

[1] Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 1996;20:707–27.
[2] Steinberg DA. critical look at the evidence for the oxidation of LDL in atherogenesis. Atherosclerosis (Suppl) 1997;131:S5–7.
[3] Heinecke JW. Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. Atherosclerosis 1998;141:1–15.
[4] Stocker R. Dietary and pharmacological antioxidants in atherosclerosis. Curr Opin Lipidol 1999;10:589–97.
[5] Jessup W, Kritharides L. Metabolism of oxidized LDL by macrophages. Curr Opin Lipidol 2000;11:473–81.
[6] Heinecke JW. Is the emperor wearing clothes? Clinical trials of vitamin E and the LDL oxidation hypothesis. Arterioscler Thromb Vasc Biol 2001;21:1261–4.
[7] Kromhout D. Serum cholesterol in cross-cultural perspective. The Seven Countries Study. Acta Cardiol 1999;54:155–8.
[8] Gey KF, Puska P, Jordan P, Moser UK. Inverse correlation between plasma vitamin E and mortality from ischaemic heart disease in cross-cultural epidemiology. Am J Clin Nutr 1991;53:326s–34s.
[9] Riemersma RA, Wood DA, Macintyre CCA, Elton RA, Gey KF, Oliver MF. Risk of angina pectoris and plasma concentrations of vitamins A, C, and E, and carotene. Lancet 1991;337:1–5.
[10] Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. Lancet 1993;342:1007–11.
[11] Hollman PC, Feskens EJ, Katan MB. Tea flavonols in cardiovascular disease and cancer epidemiology. Proc Soc Exp Biol Med 1999;220:198–202.
[12] de Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. Circulation 1999;99:779–85.
[13] Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC, Vitamin E. consumption and the risk of coronary disease in women. New Engl J Med 1993;328:1444–9.
[14] Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. New Engl J Med 1993;328:1450–6.
[15] Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y, Bostick RM. Dietary antioxidant vitamins and death from coronary heart

disease in postmenopausal women. New Engl J Med 1996;334:1156–62.
[16] Meagher EA, Barry OP, Lawson JA, Rokach J, FitzGerald GA. Effects of vitamin E on lipid peroxidation in healthy persons. J Am Med Assoc 2001;285:1178–82.
[17] Suarna C, Dean RT, May J, Stocker R. Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. Arterioscler Thromb Vasc Biol 1995;15:1616–24.
[18] Keaney JF, Jr, Simon DI, Freedman JE. Vitamin E and vascular homeostasis: implications for atherosclerosis. FASEB J 1999;13:965–75.
[19] Ruf JC. Wine and polyphenols related to platelet aggregation and atherothrombosis. Drugs Exp Clin Res 1999;25:125–31.
[20] Tsai SH, Liang YC, Lin-Shiau SY, Lin JK. Suppression of TNFα-mediated NFκB activity by myricetin and other flavonoids through downregulating the activity of IKK in ECV304 cells. J Cell Biochem 1999;74:606–15.
[21] Shaish A, Daugherty A, O'Sullivan F, Schonfeld G, Heinecke JW. β-carotene inhibits atherosclerosis in hypercholesterolemic rabbits. J Clin Invest 1995;96:2075–82.
[22] Witting P, Pettersson K, Ostlund-Lindqvist AM, Westerlund C, Wagberg M, Stocker R. Dissociation of atherogenesis from aortic accumulation of lipid hydro(pero)xides in Watanabe heritable hyperlipidemic rabbits. J Clin Invest 1999;104:213–20.
[23] Meagher EA, FitzGerald GA. Indices of lipid peroxidation in vivo: strengths and limitations. Free Radic Biol Med 2000;28:1745–50.
[24] Davi G, Alessandrini P, Mezzetti A, Minotti G, Bucciarelli T, Constantini F, Cipollone F, Bon GB, Ciabattoni G, Patrono C. In vivo formation of 8-iso-prostaglandin F2 α is increased in hypercholesterolemia. Arterioscler Thromb Vasc Biol 1997;17:3230–5.
[25] Davi G, Ciabattoni G, Consoli A, Mezzetti A, Falco A, Santarone S, Pennese E, Vitacolonna E, Bucciarelli T, Costantini F, Capani F, Patrono C. In vivo formation of 8-isoprostaglandin F2 α and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. Circulation 1999;19:224–9.
[26] Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, Strauss WE, Oates JA, Roberts LJ. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. New Engl J Med 1995;332:1198–203.
[27] Reilly M, Delanty N, Lawson JA, Fitzgerald GA. Modulation of oxidant stress in vivo in chronic cigarette smokers. Circulation 1996;94:19–25.
[28] Marangon K, Devaraj S, Tirosh O, Packer L, Jialal I. Comparison of the effect of a-lipoic and a-tocopherol supplementation on measures of oxidative stress. Free Radic Biol Med 1999;27:1114–21.
[29] Weinberg RB, VanderWerken BS, Anderson RA, Stegner JE, Thomas MJ. Pro-oxidant effect of vitamin E in cigarette smokers consuming a high polyunsaturated fat diet. Arterioscler Thromb Vasc Biol 2001;21:1029–33.
[30] O'Reilly JD, Mallet AI, McAnlis GT, Young IS, Halliwell B, Sanders TA, Wiseman H. Consumption of flavonoids in onions and black tea: lack of effect on F2-isoprostanes and autoantibodies to oxidized LDL in healthy humans. Am J Clin Nutr 2001;73:1040–4.
[31] Kritchevsky SB, Shimakawa T, Tell GS, Dennis B, Carpenter M, Eckfeldt JH, Peacher-Ryan H, Heiss G. Dietary antioxidants and carotid artery wall thickness. The ARIC Study. Atherosclerosis Risk in Communities Study. Circulation 1995;92:2142–50.
[32] Adams MR, Nakagomi A, Keech A, Robinson J, McCredie R, Bailey BP, Freedman SB, Celermajer DS. Carotid intima-media thickness is only weakly correlated with the extent and severity of coronary artery disease. Circulation 1995;92:2127–34.
[33] Azen SP, Qian D, Mack WJ, Sevanian A, Selzer RH, Liu CR, Liu CH, Hodis HN. Effect of supplementary antioxidant vitamin intake on carotid arterial wall intima-media thickness in a controlled clinical trial of cholesterol lowering. Circulation 1996;94:2369–72.
[34] MacMahon S, Sharpe N, Gamble G, Hart H, Scott J, Simes J, White H. Effects of lowering average of below-average cholesterol levels on the progression of carotid atherosclerosis: results of the LIPID Atherosclerosis Substudy, LIPID Trial Research Group. Circulation 1998;97:1784–90.
[35] MacMahon S, Sharpe N, Gamble G, Clague A, Mhurchu CN, Clark T, Hart H, Scott J, White H. Randomized, placebo-controlled trial of the angiotensin-converting enzyme inhibitor, ramipril, in patients with coronary or other occlusive arterial disease. PART-2 Collaborative Research Group. Prevention of atherosclerosis with ramipril. J Am Coll Cardiol 2000;36:438–43.
[36] Lonn E, Yusuf S, Dzavik V, Doris C, Yi Q, Smith S, Moore-Cox A, Bosch J, Riley W, Teo K. Effects of ramipril and vitamin E on atherosclerosis: the study to evaluate carotid ultrasound changes in patients treated with ramipril and vitamin E (SECURE). Circulation 2001;103:919–25.
[37] Salonen JT, Nyyssonen K, Salonen R, Lakka HM, Kaikkonen J, Porkkala-Sarataho E, Voutilainen S, Lakka TA, Rissanen T, Leskinen L, Tuomainen TP, Valkonen VP, Ristonmaa U, Poulsen HE. Antioxidant supplementation in atherosclerosis prevention (ASAP) study: a randomized trial of the effect of vitamins E and C on 3-year progression of carotid atherosclerosis. J Intern Med 2000;248:377–86.
[38] Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, Yang CS, Zheng SF, Gail M, Li GY, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. J Natl Cancer Inst 1993;85:1483–92.
[39] The α-Tocopherol, β Carotene Cancer Prevention Study Group. The effect of vitamin E and β carotene on the incidence of lung cancer and other cancers in male smokers. New Engl J Med 1994;330:1029–35.
[40] Virtamo J, Rapola JM, Ripatti S, Heinonen OP, Taylor PR, Albanes D, Huttunen JK. Effect of vitamin E and β carotene on the incidence of primary nonfatal myocardial infarction and fatal coronary heart disease. Arch Intern Med 1998;158:668–75.
[41] Rapola JM, Virtamo J, Ripatti S, Huttunen JK, Albanes D, Taylor PR, Heinonen OP. Randomised trial of α-tocopherol and β-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. Lancet 1997;349:1715–20.
[42] Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a combination of β carotene and vitamin A on lung cancer and cardiovascular disease. New Engl J Med 1996;334:1150–5.
[43] Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, Belanger C, LaMotte F, Gaziano JM, Ridker PM, Willett W, Peto R. Lack of effect of long-term supplementation with β carotene on the incidence of malignant neoplasms and cardiovascular disease. New Engl J Med 1996;334:1145–9 (see comments).
[44] PPP, (primary prevention project) low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Lancet 2001;357:89–95.
[45] Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). Lancet 1996;347:781–6.
[46] Gissi Prevenzione Investigators Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Gissi-prevenzione dietary supplementation with n-3 polyunsaturated fatty acids and

vitamin E after myocardial infarction: results of the GISSI-prevenzione trial. Lancet 1999;354:447–55.
[47] Brown M. Do vitamin E and fish oil protect against ischaemic heart disease. Lancet 1999;354:441–2.
[48] Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. New Engl J Med 2000;342:154–60.
[49] Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. New Engl J Med 2000;342:145–53.
[50] Boaz M, Smetana S, Weinstein T, Matas Z, Gafter U, Iaina A, Knecht A, Weissgarten Y, Brunner D, Fainaru M, Green MS. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. Lancet 2000;356:1213–8.
[51] Descombes E, Hanck AB, Fellay G. Water soluble vitamins in chronic hemodialysis patients and need for supplementation. Kidney Int 1993;43:1319–28.
[52] Lim PS, Chan EC, Lu TC, Yu YL, Kuo SY, Wang TH, Wei YH. Lipophilic antioxidants and iron status in ESRD patients on hemodialysis. Nephron 2000;86:428–35.
[53] Brown BG, Zhao X-Q, Chait A, Fisher LD, Cheung MC, Morse JS, Dowdy AA, Marino EK, Bolson EL, Alaupovi P, Frohlich J, Albers JJ. Simvastatin and Niacin, antioxidant vitamins, or the combination for the prevention of coronary disease. New Engl J Med 2001;345:1583–92.
[54] Johansson J, Olsson AG, Bergstrand L, Elinder LS, Nilsson S, Erikson U, Molgaard J, Holme I, Walldius G. Lowering of HDL2b by probucol partly explains the failure of the drug to affect femoral atherosclerosis in subjects with hypercholesterolemia. A Probucol Quantitative Regression Swedish Trial (PQRST) Report. Arterioscler Thromb Vasc Biol 1995;15:1049–56.
[55] Collins, R. et al. MRC/BHF Heart Protection Study www.hpsinfo.org, and presented AHA, Annaheim, CA 2001.
[56] Tribble DL. Antioxidant consumption and risk of coronary heart disease: emphasis on vitamin C, vitamin E and β-carotene. Circulation 1999;99:591–5.
[57] Witting PK, Pettersson K, Letters J, Stocker R. Site-specific antiatherogenic effect of probucol in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2000;20:e26–33.
[58] Upston JM, Terentis AC, Stocker R. Tocopherol-mediated peroxidation of lipoproteins: implications for vitamin E as a potential antiatherogenic supplement. FASEB J 1999;13:977–94.
[59] Mashima R, Witting PK, Stocker R. Oxidants and antioxidants in atherosclerosis. Curr Opin Lipidol 2001;12:411–8.
[60] Terentis AC, Thomas SR, Burr JA, Liebler DC, Stocker R. Vitamin E oxidation in human atherosclerotic lesions. Circ. Res. 2002: in press.

ANNEX E

Effects of Long-Term Daily Low-Dose Supplementation With Antioxidant Vitamins and Minerals on Structure and Function of Large Arteries

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Objective—Limited data exist from randomized trials evaluating, noninvasively, the impact of antioxidant supplementation on vascular structure and function.

Methods and Results—This is a substudy of the SU.VI.MAX Study, which is a randomized, double-blind, placebo-controlled, cardiovascular and cancer primary prevention trial. Eligible participants (free of symptomatic chronic diseases and apparently healthy) were randomly allocated to daily receive either a combination of antioxidants (120 mg vitamin C, 30 mg vitamin E, 6 mg beta carotene, 100 μg selenium, and 20 mg zinc) or placebo and followed-up over an average of 7.2±0.3 years. At the end-trial examination, the carotid ultrasound examination and carotid–femoral pulse-wave velocity (PWV) measurement were performed blindly in 1162 subjects aged older than 50 years and living in the Paris area. The percentage of subjects with carotid plaques was higher in the intervention group compared with the placebo group (35.2% versus 29.5%, $P=0.04$). Common carotid intima-media thickness (mean±SD) was not different between the 2 groups (0.70±0.08 versus 0.70±0.08 mm, $P=0.38$). Mean PWV tended to be lower (indicating less stiff aortic arteries) in the intervention group but the difference did not reach statistical significance ($P=0.13$).

Conclusion—These results suggest no beneficial effects of long-term daily low-dose supplementation of antioxidant vitamins and minerals on carotid atherosclerosis and arterial stiffness. (***Arterioscler Thromb Vasc Biol.* 2004; 24:1485-1491.)**

Key Words: atherosclerosis ■ nutrition ■ oxidant stress ■ remodeling ■ developmental biology

The results of several animal experimental and population-based epidemiological studies have suggested that enhanced lipid peroxidation is associated with atherogenesis and cardiovascular diseases.[1] Many observational studies show that a high dietary intake or high blood concentration of antioxidant vitamins are associated with reduced risk of cardiovascular diseases.[2] Dietary antioxidants are recognized to protect against lipid peroxidation. However, randomized controlled studies investigating the clinical use of antioxidant supplementation to prevent cardiovascular disease have provided conflicting, even disappointing, results.[3–8]

Plausible explanations of discrepancies include the types of the populations recruited, the timing of the intervention relative to the atherosclerotic process, the duration of the intervention, the supplementation levels used (nutritional doses versus higher doses), the number of antioxidants tested, and the type of administration used (alone versus in combination with other nutrients).[9]

To understand the mechanisms linking antioxidants to cardiovascular disease, it is necessary to assess the potential effects of antioxidant supplementation on vascular structure and function on humans. Few randomized studies have investigated this issue, and most of them were limited by the use of carotid intima-media thickness (IMT) as the only marker of vascular alterations.[10–13]

B-mode ultrasound of carotid arteries is a noninvasive, valid, and reproducible method for directly visualizing and assessing carotid structure (IMT, lumen diameter, and focal atherosclerosis [plaques]).[14–16] Noninvasive measurement of carotid–femoral pulse-wave velocity (PWV) is an easy and reproducible method of assessing aortic arterial stiffness,[17] a major component of vascular function.

In this study of 1162 subjects aged older than 50 years and living in the Paris area, we report the effects of long-term daily nutritional dose supplementation with antioxidant vitamins and minerals on carotid IMT, lumen diameter, and

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plaques and aortic stiffness assessed by B-mode ultrasound and carotid-femoral PWV.

Methods

This is a substudy of the SU.VI.MAX (SUpplémentation en VItamines et Minéraux AntioXydants) Study, which is a randomized, double-blind, placebo-controlled, primary prevention trial undertaken to determine whether supplementation with antioxidant vitamins and minerals at nutritional doses can reduce the incidence of cancers and cardiovascular diseases. The rationale, design, and methods of the study as well as characteristics of the participants have been described in detail elsewhere.[18,19] In brief, in March through July 1994, information on the objectives and outline of the study was presented in various public media, along with a call for volunteers (women aged 35 to 60, or men aged 45 to 60, living in France). Eligible individuals free of symptomatic chronic diseases and apparently healthy at baseline (judged by clinical examination) were invited to an enrollment visit during which they received a manual, along with software or paper forms, to be completed during follow-up, and were randomly allocated to receive either a combination of antioxidants (120 mg vitamin C, 30 mg vitamin E, 6 mg beta carotene, 100 μg selenium (as selenium-enriched yeast) and 20 mg zinc (as gluconate) or a matching placebo, in a single daily capsule. The rationale of using this combination and doses has been previously provided.[18] Capsules were prepared in 52 weekly packages of 7 capsules provided in a yearly box, labeled with the subjects participant number and a 10-digit lot number. Random treatment allocation was performed by block-sequence generation, stratified by gender and age-group; 13 017 eligible subjects were included to be followed-up for 7.5 years. Participants visited local SU.VI.MAX facilities each year. These visits involved the collection of blood and/or an extensive clinical examination.

At the end of follow-up, 74% of the participants reported having taken at least two-thirds of the capsules. There were no differences in capsule consumption between the groups (mean percentage of capsules taken: 79% in each). Compliance was confirmed for the intervention group by statistically significant increases in all biochemical markers of supplementation after 2 years, and after 7 years for beta carotene, vitamin C, and selenium in a subgroup of subjects (Table I, available online at http://atvb.ahajournals.org).

All subjects gave their informed written consent to the study, which was approved by the *ad hoc* ethical committees, ie, the "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale" (CCPPRB no 706 Paris-Cochin Hospital, France) and the "Commission Nationale de l'Informatique et des Libertés" (CNIL no 334641).

Eligible subjects for this specific substudy were participants living in the Paris area, aged older than 50 years in 2002, whose end-trial visit should take place in 1 facility (CNAM center). These subjects were blindly examined between January and July 2002. The ultrasound examination and PWV measurement of this side protocol were added to the standard measures performed during the end-trial visit.

Carotid Ultrasound Examination

Ultrasound examinations were performed with the use of the Aloka SSD-650, with a transducer frequency of 7.5 MHz. Acquisition, processing, and storage of B-mode images were computer-assisted with the new version of a software previously described (M'ATHS).[20]

Ultrasound examinations were performed by 2 trained technicians and the protocol, which was similar to that applied in the Aging Vascular Study (EVA Study).[21,22] Please see online Methods, available at http://atvb.ahajournals.org

PWV

This parameter is inversely proportional to the square root of the voluminal distensibility of the aorta. Carotid–femoral PWV was evaluated using 2 pressure probes. This method using an automatic device (Complior, Colson) has been extensively analyzed.[23] Please see online Methods, available at http://atvb.ahajournals.org.

Reproducibility Study

Please see online Methods, available at http://atvb.ahajournals.org

Baseline and End-Trial Risk Factors Assessment

Please see online Methods, available at http://atvb.ahajournals.org.

Data Analysis

All analyses comparing the placebo group to the intervention group were performed by intention-to-treat and standard procedures from the Statistical Analysis System (SAS, Cary, NC) were used for statistical analyses. Outcomes considered in the analyses were carotid plaques (qualitative variable), common carotid artery (CCA)–IMT, CCA–lumen diameter, and PWV (quantitative variables). Baseline and end-trial characteristics according to intervention and placebo groups were compared by t test, χ^2 test, and Fisher exact test as appropriate (results were expressed as percentages or means±SD). Multivariate adjustments for potential baseline cardiovascular risk factors and serum vitamins and minerals were performed by analyses of covariance (ANCOVA) for continuous variables and multiple logistic regression models for qualitative variables. Potential baseline cardiovascular risk factors considered in the analyses were age, sex, body mass index, hypertension (or hypertension treatment), total cholesterol, diabetes, and smoking habits. All multivariate analyses were repeated after substitution of end-trial cardiovascular risk factors for baseline cardiovascular risk factors. All reported P values are 2-tailed and $P<0.05$ was considered significant.

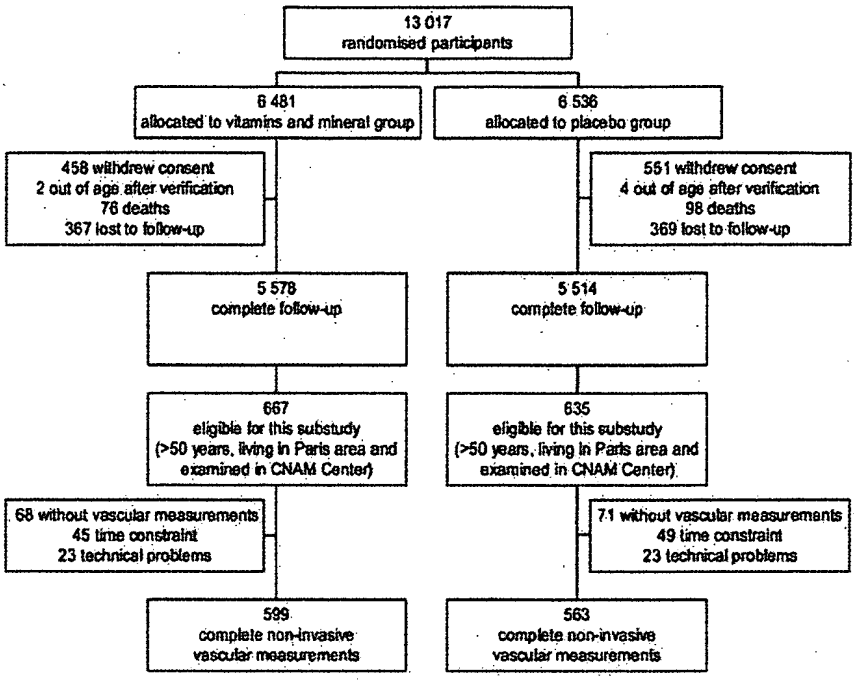
We have planned to include ≈1200 subjects. This provides, at the 0.05 significance level, at least 90% power to detect 0.03-mm differences between the 2 groups for CCA-IMT, 0.4 m/s for PWV, and 7% differences in carotid plaque prevalence.

Results

Of 1302 eligible individuals, 1162 (89.2%) have undergone complete vascular measurements (563 in the placebo group and 599 in the intervention group) (Figure). Reasons for dropout included technical problems and time constraint caused mainly by the high number of examined subjects on certain days. At baseline, there were no statistical significant differences between eligible included subjects and eligible excluded subjects concerning classical cardiovascular risk factors and serum vitamins and minerals.

The duration of follow-up was 7.2±0.3 years. The main characteristics of the 1162 study population subjects at the baseline are presented in Table 1. At baseline, there were no significant differences between treatment groups in age or any demographic, biochemical nutritional, or clinical characteristics. At the end-trial visit (Table 2), only the frequency of hypertension treatment tended to be lower in the intervention group compared with the placebo group ($P=0.11$).

The mean CCA–IMT was 0.71±0.08 mm for men and 0.69±0.07 mm for women ($P<0.001$), mean PWV was, respectively, 11.90±2.32 and 10.77±1.93 m/sec ($P<0.0001$), and the percentages of subjects with plaques were, respectively, 41.8% and 23.0% ($P<0.0001$). CCA–IMT, PWV, and carotid plaque were positively associated with baseline and end-trial values of age, body mass index, systolic blood pressure, hypertension treatment, and hypertension status. Carotid plaque was negatively associated with serum beta carotene measured at baseline. The means of baseline beta carotene in subjects without plaques and in those with plaques were, respectively, 0.60±0.39 and 0.49±0.31 μmol/L ($P<0.0001$). Subjects with carotid plaques also had a higher mean of CCA–IMT (0.72±0.08 mm versus 0.69±0.07 mm, $P<0.0001$) and a higher mean of PWV



The study design and the flow of subjects.

(11.67±2.36 m/s versus 11.18±2.11 m/s, $P<0.001$) than subjects without plaques.

Association of Carotid Plaques With Antioxidant Supplementation

The percentage of subjects with plaques was higher in the intervention group compared with the placebo group (35.2% versus 29.5%, $P=0.04$) (Table 3). Multivariate analyses (multiple logistic regression model) showed that this association was independent of sex, baseline age, and the baseline other potential risk factors including serum vitamins and minerals. The multivariate odds ratio of carotid plaques in the intervention group compared with the placebo group was 1.24 (95% CI, 1.01 to 1.52, $P=0.03$). In the multivariate model, the substitution of end-trial cardiovascular risk factors for baseline cardiovascular risk factors did not modify the results (odds ratio=1.22, 95% CI, 1.01 to 1.50, $P=0.04$). This was also the case when further adjustment for CCA–IMT and/or PWV was performed.

Analyses separately repeated in subgroups according to sex, baseline age (younger than 50 years, 50 years and older), hypertension status, smoking habits, serum vitamins, and minerals yielded similar patterns of results, and no interaction term was statistically significant (Table II, available online at http://atvb.ahajournals.org).

Associations of CCA–IMT and CCA–Lumen Diameter With Antioxidant Supplementation

Neither CCA–IMT nor CCA–lumen diameter was associated with antioxidant supplementation (Table 4). Multivariate analyses (ANCOVA) and/or analyses separately performed in each subgroup did not modify these results.

Association of PWV With Antioxidant Supplementation

Mean PWV tended to be lower in the intervention group compared with the placebo group, but the difference did not reach statistical significance (Table 4). However, women in the intervention group had significantly lower mean PWV than women who received placebo (Table 4). In men, the lack of association between PWV and antioxidant supplementation was confirmed in the multivariate analyses (ANCOVA).

In women, when multivariate analyses were performed, the adjusted mean PWV was 10.53±1.83 m/s in the intervention group and 11.05±1.91 m/s in the placebo group ($P=0.02$). When PWV values were divided into tertile categories, the multivariate odds ratio of having higher values of PWV (tertile 3) in the intervention group compared with the placebo group (provided from multiple logistic regression model) was 0.48 (95% CI, 0.26 to 0.92, $P=0.01$). Substitution of end-trial cardiovascular risk factors for baseline cardiovascular risk factors did not modify the results.

Discussion

The main findings of this large-scale study of the effects of vitamins and minerals supplementation on vascular structure and function of large arteries were as follows. First, carotid atherosclerotic plaques tend to be more frequent in the group that received antioxidants, independently of conventional cardiovascular risk factors and serum antioxidants. Second, PWV tends to be lower in the intervention group, and the differences between groups were more pronounced and statistically significant in women. Third, neither CCA–IMT nor CCA–lumen diameter seemed to be modified by antioxidant supplementation.

TABLE 1. Baseline Population Characteristics According to the Placebo and the Intervention Groups

	Placebo Group (n=563)	Intervention Group (n=599)	*P*
Age, years	52.6±4.7*	52.7±4.7	0.56
Male, %	49.6	50.8	0.68
BMI, kg/m²	24.8±3.7	24.9±3.5	0.67
Smoking habits, %			0.53
Never	50.5	48.2	
Ex-smoker	37.6	37.8	
Smoker	11.9	14.0	
Systolic BP, mm Hg	125.8±14.8	125.2±15.2	0.53
Diastolic BP, mm Hg	81.3±9.0	81.3±9.8	0.92
Hypertensive medication, %	11.5	10.8	0.68
Hypertension, %	20.1	19.5	0.79
Total cholesterol, mmol/l	6.1±1.0	6.1±1.0	0.90
Lipid lowering drugs, %	6.6	5.7	0.52
Diabetes, %	3.7	3.8	0.91
Plasma beta-carotene, mmol/l	0.6±0.4	0.6±0.4	0.30
Plasma vitamin E, mmol/l	31.7±7.1	31.6±7.5	0.83
Plasma vitamin C, mg/l	9.6±3.7	10.1±5.1	0.12
Plasma selenium, mmol/l	1.1±0.2	1.1±0.2	0.74
Plasma zinc, mmol/l	13.3±1.8	13.2±1.9	0.46
Dietary intake**			
Energy intake			
total (Kcal/j)	1902±521	1919±496	0.60
without alcohol (Kcal/j)	1784±472	1794±457	0.74
Lipids (g/j)	77.8±23.1	78.3±22.8	0.73
Proteins (g/j)	83.6±22.2	83.9±20.6	0.83
Glucides (g/j)	187.5±57.0	188.5±56.7	0.77
Alcohol consumption (g/j)	16.8±17.7	17.8±18.4	0.42
Beta-carotene (μg/j)	3494±2017	3482±2055	0.77
Vitamin C (mg/j)	95.1±40.8	92.0±42.1	0.22
Vitamin E (mg/j)	9.8±3.7	9.9±3.5	0.88

BMI indicates body mass index; BP, blood pressure, CHD, coronary heart disease.

*Mean±SD; **Data from subjects with at least 3 24-hour diet recalls (513 in the placebo group and 553 in the intervention group).

Our present study is a substudy of the SU.VI.MAX Study, a randomized, double-blind, placebo-controlled, primary prevention trial designed to test the efficacy of daily supplementation with antioxidant vitamins and minerals. Compared with the other randomized trials, several particularities of this study could be noted. Three vitamins (C, E, and beta carotene) and 2 minerals (selenium and zinc) were combined at nutrition-like doses (1 to 3 times the daily recommended dietary allowances) and were supplemented in subjects not selected because of cardiovascular risk factors. The rates of cardiovascular clinical events after a median follow-up of 7.5 years were not different between the intervention and placebo groups.[24] These results were in agreement with those of

TABLE 2. End-Trial Population Characteristics According to the Placebo and the Intervention Groups

	Placebo Group (n=563)	Intervention Group (n=599)	*P*
BMI, kg/m²	25.2±3.8	25.1±3.5	0.70
Smoking habits, %			0.30
Never	46.9	45.1	
Ex-smoker	41.8	40.5	
Smoker	11.3	14.4	
Systolic BP, mm Hg	129.4±17.3	128.4±16.6	0.34
Diastolic BP, mm Hg	77.4±9.6	77.0±9.3	0.58
Hypertensive medication, %	21.0	17.4	0.11
Hypertension, %	34.6	32.2	0.40
Total cholesterol, mmol/l	5.7±0.9	5.7±0.9	0.87
Lipid lowering drugs, %	20.2	19.6	0.76
HDL cholesterol, mmol/l	11.5±0.4	1.6±0.4	0.67
LDL cholesterol, mmol/l	3.7±0.9	3.7±0.8	0.70
Diabetes, %	6.8	6.3	0.70
CHD,** %	1.4	2.0	0.80

BMI indicates body mass index; BP, blood pressure, CHD, coronary heart disease.

*Mean±SD; **Myocardial infarction or unstable angina.

several trials suggesting a lack of beneficial effects of vitamins supplementation (especially vitamin E and beta carotene) in cardiovascular primary and secondary preventions.[3–8]

Few randomized studies using noninvasive cardiovascular parameters like IMT have been previously published and their results were controversial. In the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study, which was a randomized trial of the effect of vitamin E (272 IU per day) and slow-release vitamin C (500 mg per day) on 3-year and 6-year progressions of carotid atherosclerosis in 520 hypercholesterolemic patients, the supplementation with

TABLE 3. Carotid Plaques According to the Placebo and the Intervention Groups

	Placebo Group (n=563)	Intervention Group (n=599)	*P**
Prevalence of plaques, %	29.5	35.2	0.04
No. of sites with plaques, %			0.09
No plaques	70.5	64.8	
Unilateral plaque	19.9	22.7	
Bilateral plaques	9.6	12.5	
No. of segments with plaques,** %			0.18
No plaques	70.5	64.8	
One segment	19.7	22.4	
Two segments	9.1	11.7	
≥Three segments	0.7	1.2	

*Calculated by χ^2 test for prevalence of plaques and No. of sites with plaques and by exact fisher test for No. of segments with plaques.

**Left and/or right CCA segments and/or CB-ICA segments.

TABLE 4. Mean Values (±SD) of CCA-IMT, CCA-Lumen Diameter, and Pulse Wave Velocity According to the Placebo and the Intervention Groups

	Placebo Group (n=563)	Intervention Group (n=599)	P
CCA-IMT, mm			
All subjects	0.70±0.08	0.70±0.08	0.38
Men	0.72±0.08	0.71±0.08	0.28
Women	0.69±0.07	0.69±0.07	0.84
CCA-lumen diameter, mm			
All subjects	6.91±0.64	6.94±0.63	0.41
Men	7.20±0.62	7.25±0.60	0.29
Women	6.62±0.51	6.62±0.50	0.94
Pulse Wave Velocity, m/sec			
All subjects	11.44±2.18	11.24±2.23	0.13
Men	11.94±2.37	11.87±2.30	0.70
Women	10.95±1.87	10.60±1.97	0.03

combination of vitamin E and vitamin C slowed carotid IMT progression in men but not in women.[10,11] The rate of progression of IMT was unaffected by the use of either antioxidant alone in men. In the Study to Evaluate Carotid Ultrasound Changes in patients treated with ramipril and vitamin E (SECURE), 400 IU of vitamin E daily had no detectable effect on carotid IMT progression after 4.5 years (on average) of follow-up in a randomized study of 637 high-risk men and women aged 55 years or older at baseline.[12] In the Vitamin E Atherosclerosis Prevention Study (VEAPS), the supplementation of vitamin E (400 IU per day) over a 3-year period in 162 healthy men and women at low risk for cardiovascular disease increased (with borderline statistical significance) the progression of carotid IMT compared with 170 subjects randomized to placebo.[13]

Carotid IMT is commonly used as a surrogate marker of atherosclerosis. However, B-mode ultrasonography is unable to differentiate the intimal from the medial layer, so the anatomic structure involved in the arterial wall thickening cannot be determined.[22] Carotid intima-media thickening may result from an atherosclerosis process affecting intima and from hypertension- and arterial stiffness-associated medial vascular hypertrophy.[25] In our study, the modest deleterious effects of supplementation on confirmed atherosclerosis end-point (plaque) would lead to increasing intima thickness, and the trend for beneficial effects on arterial stiffness may lead to decreasing media thickness. These opposite effects on arterial thickness may explain, in part, the lack of association between supplementation and CCA–IMT.

In the present report, subjects with vitamin and mineral supplementation had higher carotid plaques. One hypothesis is that the associations of vitamin supplementation with carotid plaques might be mediated by the pro-oxidant effects of vitamin E promoting intimal lipid peroxidation and atherosclerosis.[26,27] In fact, vitamin E can have antioxidant, neutral, or pro-oxidant activity, and this more complex function may be then reflected in the inconclusive results of vitamin E intervention studies of atherosclerosis in animals and in humans.[26] Nevertheless, further studies are needed to confirm our results and to better-understand the reasons for the possible increased risk of atherosclerotic plaque with vitamin supplementation.

In the literature, many different ultrasound protocols have been used, and there is no consensus on carotid examination in epidemiological studies and clinical trials. In the present study, we used a methodological approach for carotid imaging that clearly differentiates between plaque and diffuse intima-media thickening. We have previously reported that the 2 types of lesions were interrelated,[22] but some factors could be specifically associated with increased IMT alone or with plaques alone.[25,28] Other investigators have measured maximum (or mean of maximum) of carotid IMT from one and/or several segments including sites with plaques in their measurements.[14,15,29] When we constructed, a posteriori, a variable allowing maximum CCA–IMT values for subjects without plaques and maximum plaque thickness for those with plaques at common carotids, this variable tended to be higher in the intervention group compared with the placebo group (0.84±0.14 mm versus 0.79±0.15 mm, $P=0.12$). In our protocol, an optimal transverse image (one for each side: left and right) at the position of the thickest part (far or near wall) of the intima-media complex (visually judged) was also captured and IMT was measured off-line. The maximum carotid thickness was higher in the intervention group compared with the placebo group (1.06±0.20 mm versus 0.98±0.20 mm, $P<0.03$) and the percentages of subjects with maximum IMT ≥1 mm were, respectively, 41.6% and 34.5% ($P<0.02$). Although these results support our main results concerning the association between carotid plaques and supplementation groups, direct comparisons with other studies are not easy and the interpretation of results provided by different studies may be dependent on the methodology used to assess the IMT, especially on the site of measurement and the inclusion or not of atherosclerotic plaques in the measurement interval.

To our knowledge, this is the first study that reports the effects of long-term daily antioxidant supplementation on arterial stiffness. It has been previously shown that short-term supplementation (8 weeks) of vitamin E (400 IU daily) improved arterial compliance in 28 volunteer middle-aged men and women.[30] In 30 patients with type 2 diabetes, supplementation of 500 mg/d of vitamin C for 1 month also improved arterial stiffness.[31] In our study, the PWV values were lower in the intervention group compared with the placebo group in women, indicating better arterial compliance and function. This result should be interpreted with caution because it was obtained from a subgroup analysis. The explanations of the differential association of antioxidant supplementation with PWV in men and women are unclear and the mechanisms linking antioxidants to arterial function are largely unknown. Age and blood pressures (or hypertension) are the strongest determinants of arterial stiffness.[25] At the end-trial visit, the frequency of hypertension treatment tended to be lower in the intervention group. However, adjustment for hypertension status at baseline visit or at end-trial visit (or hypertension treatment) did not modify our results, suggesting that the observed associations were largely

independent of blood pressure. It was speculated that the potential beneficial vitamins effects on arterial stiffness might be the results of improved endothelial vasodilator function or perhaps an effect on vascular smooth muscle cell proliferation.[32,33]

Although carotid plaques and PWV were positively associated, differential relationships of the parameters with antioxidant supplementation were observed. Atherosclerosis and arterial stiffness are to some extent related, but they are 2 distinct physiopathological processes affecting arterial structure. Our results may indicate that the potential mechanisms linking antioxidants to plaques are not mediated by arterial stiffness (and vice versa).

Several limits to our study should be noted. Carotid ultrasound and PWV examinations were not performed at baseline; therefore, progression rates of vascular parameters could not be determined. We do not think that this fact would have markedly modified our results and conclusions. Baseline cardiovascular risk factors were very well-balanced between intervention and placebo groups. In addition, even if the study may be considered as an observational investigation, the major advantage, compared with classical epidemiological studies on this issue, is that supplementation of antioxidants was randomly assigned and our results cannot then be confounded by indication bias. The classical epidemiological studies on this issue have been criticized by the fact that the use of diets rich in antioxidants and/or the use of vitamin supplements may be just markers that identify populations with higher health awareness and with healthy lifestyle behaviors, possibly entirely independent of antioxidant intake.[34] Nevertheless, the possibility that our results could have been obtained by chance and/or might at least partially reflect differences between the 2 groups in carotid plaques and/or in PWV at baseline cannot be completely excluded.

One could argue that the compliance, which was similar to those reported in other comparable vitamins trials,[11,12] was relatively low. However, the adherence rate can be considered as acceptable because our study is a very long-term primary prevention trial conducted in apparently healthy subjects. In addition, an increase in biological markers of vitamins and minerals, except vitamin E and zinc, in the intervention group were observed over time. We could not completely exclude that the doses of vitamin E and zinc were not sufficient to induce arterial modifications. Selection bias to undergo the final examination (because of the presence of coronary heart disease) might have been occurred. However, the number of subjects with coronary heart disease was low and not different between the 2 groups, and the exclusion of these subjects from statistical analyses yielded very similar results to those conducted in the whole population (data available from authors). In addition, the percentage of carotid plaques and the distributions of CCA–IMT and PWV in the placebo group were comparable to those reported in other French populations at similar age.[21,25,35] The definition of carotid plaques may be difficult by B-mode ultrasound and varies considerably across studies. When we defined, a posteriori, the plaques as a localized protrusion of the vessel wall into the lumen with a thickness of $\geq$1.2 mm instead of $\geq$1 mm, the prevalences of plaques were 32.6% in the intervention group and 28.1% in the placebo group (P=0.08). For a thickness of $\geq$1.4 mm, they were, respectively, 30.7% and 26.0% (P=0.07). Only common carotid IMT was systematically measured in our study. However, there are large variations in IMT according to the arterial site. The internal carotid artery and the bifurcation show greater IMT and more pronounced right skewness than the CCA.[36] However, assessing and quantification of the IMT in the internal carotid artery and the bifurcation are more difficult for various technical and methodological reasons (tortuosity, proximity to the mandible, reproducibility, etc).[37] Good-quality images of the far wall of the straight part of the CCA are easy to obtain and IMT can be reliably measured in nearly all subjects.

In conclusion, the results of this large-scale study suggest no marked beneficial effects of long-term daily low-dose supplementation of antioxidant vitamins and minerals on carotid structure and arterial stiffness.

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References

1. Kritharides L, Stocker R. The use of antioxidant supplements in coronary heart disease. *Atherosclerosis*. 2002;164:211–219.
2. Jialal I, Devaraj S. Antioxidants and atherosclerosis: don't throw out the baby with the bath water. *Circulation*. 2003;107:926–928.
3. GISSI-Prevenzione trial (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet*. 1999;354:447–455.
4. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med*. 1994;330:1029–1035.
5. Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, Belanger C, LaMotte F, Gaziano JM, Ridker PM, Willett W, Peto R. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms andcardiovascular disease. *N Engl J Med*. 1996;334:1145–1149.
6. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet*. 2002;360:7–22.
7. Collaborative Group of the Primary Prevention Project. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. *Lancet*. 2001;357:89–95.
8. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med*. 2000;342:154–160.
9. Hercberg S, Galan P, Preziosi P, Alfarez MJ, Vazquez C. The potential role of antioxidant vitamins in preventing cardiovascular diseases and cancers. *Nutrition*. 1998;14:513–520.
10. Salonen JT, Nyyssonen K, Salonen R, Lakka HM, Kaikkonen J, Porkkala-Sarataho E, Voutilainen S, Lakka TA, Rissanen T, Leskinen L, Tuomainen TP, Valkonen VP, Ristonmaa U, Poulsen HE. Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study: a randomized trial of the effect of vitamins E and C on 3-year progression of carotid atherosclerosis. *J Intern Med*. 2000;248:377–386.
11. Salonen RM, Nyyssonen K, Kaikkonen J, Porkkala-Sarataho E, Voutilainen S, Rissanen TH, Tuomainen TP, Valkonen VP, Ristonmaa U, Lakka HM, Vanharanta M, Salonen JT, Poulsen HE. Six-year effect of combined vitamin

C and E supplementation on atherosclerotic progression: the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study. *Circulation*. 2003;107:947–953.
12. Lonn E, Yusuf S, Dzavik V, Doris C, Yi Q, Smith S, Moore-Cox A, Bosch J, Riley W, Teo K. Effects of ramipril and vitamin E on atherosclerosis: the study to evaluate carotid ultrasound changes in patients treated with ramipril and vitamin E (SECURE). *Circulation*. 2001;103: 919–925.
13. Hodis HN, Mack WJ, LaBree L, Mahrer PR, Sevanian A, Liu CR, Liu CH, Hwang J, Selzer RH, Azen SP. VEAPS Research Group. Alpha-tocopherol supplementation in healthy individuals reduces low-density lipoprotein oxidation but not atherosclerosis: the Vitamin E Atherosclerosis Prevention Study (VEAPS). *Circulation*. 2002;106:1453–1459.
14. Crouse JR, Goldbourt U, Evans G, Pinsky J, Sharrett AR, Sorlie P, Riley W, Heiss G, ARIC Investigators. Arterial enlargement in the Atherosclerosis Risk in Communities (ARIC) Cohort: in vivo quantification of carotid arterial enlargement. *Stroke*. 1994;25:1354–1359.
15. O'Leary DH, Polak JF, Wolfson SK, Bond MG, Bommer W, Sheth S, Psaty BM, Sharrett AR, Manolio TA on behalf of the CHS Collaborative Research Group. Use of sonography to evaluate carotid atherosclerosis in the elderly. The Cardiovascular Health Study. *Stroke*. 1991;22: 1155–1163.
16. Joakimsen O, Bonaa KH, Stensland-Bugge E. Reproducibility of ultrasound assessment of carotid plaque occurrence, thickness, and morphology. The Tromso Study. *Stroke*. 1997;28:2201–2207.
17. Asmar RG, Topouchian JA, Benetos A, Sayegh FA, Mourad JJ, Safar ME. Non-invasive evaluation of arterial abnormalities in hypertensive patients. *J Hypertens*. 1997;15(Suppl 2):S99–S107.
18. Hercberg S, Galan P, Preziosi P, Roussel A-M, Arnaud J, Richard M-J, Malvy D, Paul-Dauphin A, Briançon S, Favier A. Background and rationale behind the SU.VI.MAX Study, a prevention trial using nutritional doses of a combination of antioxidant vitamins and minerals to reduce cardiovascular diseases and cancers. *Int J Vit Nutr Res*. 1997; 68:3–20.
19. Hercberg S, Preziosi P, Briançon S, Galan P, Paul-Dauphin A, Malvy D, Roussel A-M, Favier A. A primary prevention trial of nutritional doses of antioxidant vitamins and minerals on cardiovascular diseases and cancers in general population: The SU.VI.MAX Study. Design, methods and participant characteristics. *Control Clin Trials*. 1998;19:336–351.
20. Touboul PJ, Prati P, Scarabin PY, Adrai V, Thibout E, Ducimetière P. Use of monitoring software to improve the measurement of carotid wall thickness by B-mode imaging. *J Hypertens*. 1992;10(suppl 5):S37–S41.
21. Zureik M, Touboul PJ, Bonithon-Kopp C, Courbon D, Berr C, Leroux C, Ducimetiere P. Cross-sectional and 4-year longitudinal associations between brachial pulse pressure and common carotid intima-media thickness in a general population. The EVA Study. *Stroke*. 1999;30: 550–555.
22. Zureik M, Ducimetière P, Touboul PJ, Courbon D, Bonithon-Kopp C, Berr C, Magne C. Common carotid intima-media thickness predicts occurrence of carotid atherosclerotic plaques: longitudinal results from the Aging Vascular Study (EVA) study. *Arterioscler Thromb Vasc Biol*. 2000;20:1622–1629.
23. Asmar R, Benetos A, Topouchian J, Laurent P, Pannier B, Brisac AM, Target R, Levy BI. Assessment of arterial distensibility by automatic pulse wave velocity measurement. Validation and clinicalapplication studies. *Hypertension*. 1995;26:485–490.
24. Hercberg S, Galan P, Preziosi P, Bertrais S, Mennen L, Malvy D, Roussel AM, Favier A, Briancon S. The SU.VI.MAX study: a randomised, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Arch Intern Med*. (in press).
25. Zureik M, Temmar M, Adamopoulos C, Bureau JM, Courbon D, Thomas F, Bean K, Touboul PJ, Ducimetiere P, Benetos A. Carotid plaques, but not common carotid intima-media thickness, are independently associated with aortic stiffness. *J Hypertens*. 2002;20:85–93.
26. Stocker R. The ambivalence of vitamin E in atherogenesis. *Trends Biochem Sci*. 1999;24:219–223.
27. Heinecke JW. Is the emperor wearing clothes? Clinical trials of vitamin E and the LDL oxidation hypothesis. *Arterioscler Thromb Vasc Biol*. 2001;21:1261–1264.
28. Zureik M, Touboul PJ, Bonithon-Kopp C, Courbon D, Ruelland I, Ducimetiere P. Differential association of common carotid intima-media thickness and carotid atherosclerotic plaques with parental history of premature death from coronary heart disease : the EVA study. *Arterioscler Thromb Vasc Biol*. 1999;19:366–371.
29. Bots ML, Evans GW, Riley WA, Grobbee DE. Carotid intima-media thickness measurements in intervention studies: design options, progression rates, and sample size considerations: a point of view. *Stroke*. 2003;34:2985–2994.
30. Mottram P, Shige H, Nestel P. Vitamin E improves arterial compliance in middle-aged men and women. *Atherosclerosis*. 1999;145:399–404.
31. Mullan BA, Young IS, Fee H, McCance DR. Ascorbic acid reduces blood pressure and arterial stiffness in type 2 diabetes. *Hypertension*. 2002;40: 804–809.
32. Skyrme-Jones RA, O'Brien RC, Berry KL, Meredith IT. Vitamin E supplementation improves endothelial function in type I diabetes mellitus: a randomized, placebo-controlled study. *J Am Coll Cardiol*. 2000; 36:94–102.
33. Boscoboinik D, Szewczyk A, Azzi A. Alpha-tocopherol (vitamin E) regulates vascular smooth muscle cell proliferation and protein kinase C activity. *Arch Biochem Biophys*. 1991;286:264–269.
34. Lonn E. Do antioxidant vitamins protect against atherosclerosis? The proof is still lacking. *J Am Coll Cardiol*. 2001;38:1795–1798.
35. Zureik M, Bureau JM, Temmar M, Adamopoulos C, Courbon D, Bean K, Touboul PJ, Benetos A, Ducimetière P. Echogenic carotid plaques are associated with aortic arterial stiffness in subjects with subclinical carotid atherosclerosis. *Hypertension*. 2003;41:519–527.
36. O'Leary DH, Polak JF, Kronmal RA, Kittner SJ, Bond MG, Wolfson Jr SK, Bommer W, Price TR Gardin JM, Savage PJ on behalf of the CHS Collaborative Research Group. Distribution and correlates of sonographically detected carotid artery disease in the Cardiovascular Health Study. *Stroke*. 1992;23:1752–1760.
37. Crouse JR, Craven TE, Hagaman AP, Bond MG. Association of coronary disease with segment-specific intimal-medial thickening of the extracranial carotid artery. *Circulation*. 1995;92:1141–1147.